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Original Research

Immunosuppressants and Ischemic Postconditioning in the Management of Brain Ischemia in Rats: The Role of Pharmacologic and Nonpharmacologic Treatments

Duygun Altıntaş Aykan[1], Buket Tuğan Yıldız[2], Ülkü Kazancı[3], Muhammed Seyithanoğlu[4], Tuba Koca[5], Alper Ural[6]

[1]Department of Pharmacology, Kahramanmaraş Sütçü İmam University School of Medicine Kahramanmaraş, Turkey

[2]Department of Neurology, Kahramanmaraş Sütçü İmam University School of Medicine Kahramanmaraş, Turkey

[3]Department of Pathology, Kahramanmaraş Sütçü İmam University School of Medicine Kahramanmaraş, Turkey

[4]Department of Biochemistry, Kahramanmaraş Sütçü İmam University School of Medicine Kahramanmaraş, Turkey

[5]Department of Physical Medicine and Rehabilitation, Kahramanmaraş Sütçü İmam University School of Medicine Kahramanmaraş, Turkey

[6]Department of Plastic and Reconstructive Surgery, Kahramanmaraş Sütçü İmam University School of Medicine Kahramanmaraş, Turkey

ABSTRACT

Objective: Ischemic postconditioning in limb skeletal muscle may decrease the size of the cerebral hypoperfused area after stroke. We compared the effects of ischemic postconditioning with the effects of the pharmacologic agents infliximab and leflunomide in the management of stroke.

Methods: Thirty-two rats were divided into four groups: postconditioning, infliximab, leflunomide, and saline (control). Global cerebral ischemia was induced by clamping the bilateral common carotid arteries for 20 min, and subsequently reperfusion was allowed for 2 h. Rats in the infliximab group received 7 mg/kg of infliximab immediately after and 6 h after the induction of stroke. Rats in the leflunomide group received 10 mg/kg of leflunomide immediately after and 6 h after the induction of stroke. In the postconditioning group, the unilateral limb muscle was clamped for 180 min immediately after the induction of stroke, and subsequently reperfusion was allowed for 120 min. Rats in the control group received saline immediately after and 6 h after the induction of stroke. Glutathione peroxidase, malondialdehyde, and ischemia-modified albumin were measured, and histopathologic evaluation of cerebral tissue was performed.

Results: The area of hemorrhage was significantly decreased in the infliximab group. Loss of the gray matter–white matter boundary was significantly decreased in the infliximab and leflunomide groups. Brain glutathione peroxidase was significantly increased in the infliximab group. There were no significant differences between the infliximab, leflunomide, and postconditioning groups and the control group in serum malondialdehyde and ischemia-modified albumin

Conclusion: Immunosuppression by infliximab and leflunomide, but not ischemic postconditioning, may attenuate brain ischemia–reperfusion injury. Although the curative effects of postconditioning treatment on brain injury were documented, the lengths of the postconditioning cycles are important for its efficacy.

Keywords: Brain ischemia, infliximab, ischemic postconditioning, leflunomide

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ORCID IDs of the authors: D.A.A. 0000-0001-8224-4006; B.T.Y. 0000-0001-6783-2336; Ü.K. 0000-0003-3769-1338; M.S. 0000-0002-8027-7549; T.K. 0000-0002-4596-858X; A.U. 0000-0001-8135-6444.

Corresponding Author: Duygun Altıntaş Aykan **E-mail:** altintasduygun_dr@yahoo.com

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INTRODUCTION

Ischemic stroke is a major health problem due to its high mortality and morbidity (1). Cerebral ischemia initiates numerous molecular events triggered by the energy deficit along with decreased cerebral perfusion. In cerebral ischemia, energy metabolites related to glucose metabolism, such as *adenosine* 5›-*triphosphate* (ATP) and phosphocreatine, are decreased and lactate levels are increased. This results in a metabolic imbalance. The reduction in ATP increases membrane depolarization and permeability, leading to increased levels of sodium, calcium, and chloride ions inside the cell and of potassium ions outside the cell. Glutamate activates *N-methyl-D-aspartate* (NMDA) channels, resulting in increased levels of intracellular calcium. Overactivation of NMDA receptors also contributes to the initiation of apoptosis. Previous studies have investigated the reduction or slowing down of this apoptotic process by medications and its recovery by reperfusion (2).

One of the most important results of the increase in intracellular calcium in cerebral ischemia is the release of free radicals by the formation of oxidants and subsequent activation of the *nitric oxide* synthase (NOS) pathway. Several methods have been used to determine the transcriptional factors implicated in the hypoxic or ischemic brain (3).

Previous studies reported that experimental animals exposed to short-term hypoxia were more resistant to cerebral ischemia and that ischemic preconditioning might be protective in ischemic brain injury models. According to these studies, short-term hypoperfusion had a neuroprotective effect against long-term ischemic injury (4). However, the risks associated with ischemic preconditioning of brain tissue and its very narrow therapeutic range limit the use of this method in humans (5). In contrast to ischemic preconditioning, cerebral protection by ischemic postconditioning is a relatively recent model. However, data regarding the protective mechanism of postconditioning against cerebral ischemia are very limited.

We hypothesized that ischemic postconditioning of skeletal muscle might ameliorate the hypoperfused penumbra in existing brain ischemia. We conducted a study to compare the effects of ischemic postconditioning on hypoperfused cerebral tissue with the effects of two immunosuppressive agents: infliximab and leflunomide. Infliximab is an inhibitor of *tumor necrosis factor*-α (TNF-α), which is one of the most important factors in initiating cell death in cerebral ischemia. Leflunomide, an inhibitor of pyrimidine synthesis and a derivative of isoxazole, has immunosuppressive and anti-inflammatory properties.

Thus, the aim of this study was to evaluate the effects of infliximab, leflunomide, and ischemic postconditioning on the amelioration of ischemic regions in the cerebrum.

Main Points:

- Short-term hypoxia and ischemic preconditioning were reported be protective in ischemic brain injury models previously.
- Risks associated with ischemic preconditioning of brain tissue and its very narrow therapeutic range limit the use of this method in humans.
- Immunosuppression by infliximab and leflunomide resulted in significant reduction in brain ischemia after vascular occlusion.
- The model of Ischemic postconditioning may be also effective and reliable. However, the lengths of the postconditioning cycles are important for its efficacy.

METHODS

The study was carried out in the Kahramanmaraş Sütçü İmam University Medical Faculty Pharmacology Department. All experiments were conducted in strict accordance with the National Institutes 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 of Kahramanmaraş Sütçü İmam University (file no. 2018/07/02, approval date 10.04.2018). The rats were housed and maintained at a temperature of 22°C, a relative humidity of 60±5%, and a 12-h/12-h light/dark cycle with access to food and water *ad libitum*.

Drugs and Chemicals

Leflunomide (Abdi Ibrahim, Turkey) was prepared at 10 mg/kg, mixed with drinking water, and given twice in a volume of 1 mL/kg by oral gavage. Infliximab (Merck Sharp Dohme, Singapore) was prepared at 7 mg/kg, dissolved in sterile 0.9% saline, and administered intraperitoneally twice in a volume of 1 mL/kg. At the end of the experiment, the rats were anesthetized with ketamine HCl (80 mg/kg) and xylasine (10 mg/kg).

Induction of Global Cerebral Ischemia and Postconditioning in Skeletal Muscle

Thirty-two Wistar albino rats were equally and randomly divided into four groups (*n*=8 per group): group 1, postconditioning (PC); group 2, infliximab (Infx); group 3, leflunomide (Lef); and group 4, saline (control). All rats underwent induced global cerebral ischemia by occlusion of the bilateral common carotid arteries, as described by Zhou et al. (6). Briefly, the rat was fixed on an operating table in a supine position after being anesthetized with ketamine HCl (80 mg/kg) and xylasine (10 mg/kg). After superficial microdissection with a midline incision, a deep microdissection proceeded toward the common carotid arteries. Both common carotid arteries were exposed through the midline incision in the neck and temporarily clipped for 20 min with cross-clamps. After 20 min, the clips were removed to restore the blood flow for recirculation, and reperfusion was allowed for 2 h. Control rats underwent the same surgical procedure and occlusions, but the drugs were not administered.

In the PC group, ischemic postconditioning was induced by applying a tourniquet to the upper third of the right leg, as described by Ergün et al. (7), for 180 min immediately after reperfusion by the carotids. The ischemic period of the limb muscle was selected to be 180 min, so that the distal pulse of the compressed limb could not be taken. After 180 min, limb muscle reperfusion was allowed for 120 min. Rats in the Infx group received 7 mg/kg of infliximab intraperitoneally twice, immediately after and 6

h after reperfusion by the carotids. Rats in the Lef group received 10 mg/kg of leflunomide orally twice, immediately after and 6 h after reperfusion by the carotids. Rats in the saline (control) group received saline intraperitoneally twice, immediately after and 6 h after reperfusion by carotids.

Verification of Skeletal Muscle Ischemia

Skeletal muscle ischemia in the compressed limb was verified by determining elevated levels of serum creatine kinase (CK) and lactate dehydrogenase (LDH) due to muscle destruction. Reperfusion was verified by regaining the distal pulse and normalization of skin color. Serum CK and LDH activities were evaluated spectrophotometrically by a Siemens ADVİA 1800 Chemistry Autoanalyser (Siemens Healthcare GmbH, Germany).

Collection of Cerebral Tissue Samples

At the end of the experiments, the rats were sacrificed under general anesthesia, and the brains were removed, immersed in fixative (10% formalin solution) for 24 h, and embedded in paraffin, monitored the tissue processing by autotechnicon device (Leica ASP 300, Germany), and microsectioned at a thickness of 4 µm (Leica Microtome RM 2145, Germany). The brain samples, stained with hematoxylin and eosin, were examined for infarct-associated morphological changes (Olympus BX53 polarizing microscope, Germany).

Evaluation of Histopathological Ischemic Changes in Brain Tissue

An overall score of the severity of cerebral tissue damage was semiquantitatively assessed taking account of vascularization (proliferation of vascular structures secondary to healing), macrophages (inflammatory component increased in wound healing), necrosis (infarct-induced coagulation necrosis), edema (in the interstitial area during the stages of inflammation), hemorrhage (red blood cells in the extravascular area), loss of the gray matter–white matter boundary (loss of the distinction between gray and white matter in the brain), neutrophilic infiltration (increased numbers of neutrophils in the area surrounding necrosis), and small red neurons (changes in glial cell morphology affected by ischemia). The severity of cerebral tissue damage was semiquantitatively scored as 0 (normal), 1.0 (mild), 2.0 (moderate), or 3.0 (severe).

Assessment of Hypoxia-Induced Oxidant Markers in Brain Tissue and Serum

Glutathione peroxidase (GSH-Px), malondialdehyde (MDA), and ischemia-modified albumin (IMA) levels were investigated in serum and tissue homogenates. Blood was collected from the rats by cardiac puncture. Serum was obtained by centrifugation at 4000 rpm for 10 min. Brain tissue samples were taken quickly and washed in cold saline. Brain tissues were homogenized with cold 0.15 M KCl (10%, w/v). Tissue homogenates were centrifuged at 600×*g* for 10 min at 4°C to remove crude fractions. The supernatants were then centrifuged at 10,000×*g* for 20 min to obtain the postmitochondrial fraction. GSH-Px activities were determined in the postmitochondrial fraction. MDA levels in homogenates and serum were determined using thiobarbituric acid. IMA levels in serum were colorometrically measured. GSH-Px activity was measured with cumene hydroperoxide as substrate. In this method, GSH-Px activity was coupled to the oxidation of NADPH by glutathione reductase, and the oxidation of NADPH was followed spectrophotometrically at 340 nm at 37°C. The results were calculated using extinction coefficient (6.22×10^3/M cm).

Statistical Analysis

The data were presented as arithmetic means and standard deviations. In order to apply parametric tests, the Kolmogorov–Smirnov test was used to determine whether the results were normally distributed and whether the variances were homogeneous. For multiple groups, analysis of variance with a post hoc Tukey's test for the significance of differences was used for normally distributed data. The Kruskal–Wallis test with the Mann–

Figure 1. Serum creatine kinase (CK) levels after compression of limb skeletal muscle

*Significant increase in the postconditioning (PC) group compared with the control group (p=0.014)

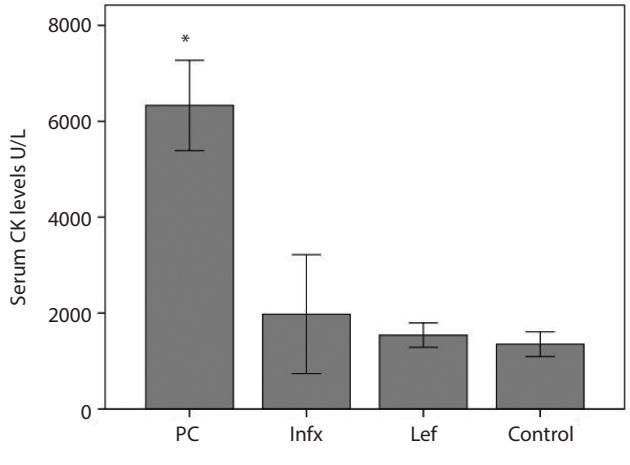
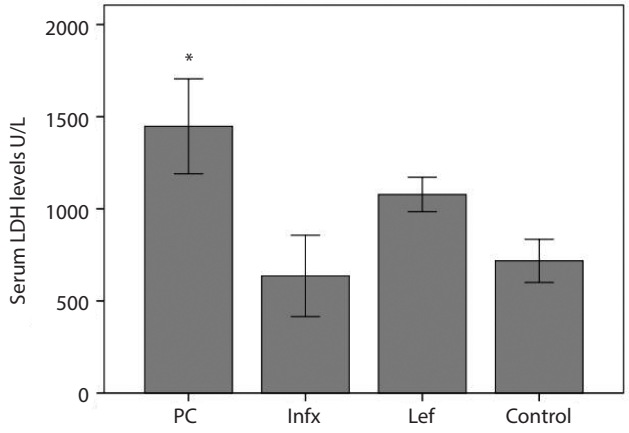


Figure 2. Serum lactate dehydrogenase (LDH) levels after limb skeletal muscle compression

*Significant increase in the postconditioning (PC) group compared with the control group (p=0.013)



Whitney U test under Bonferroni correction was used for analysis of non-normally distributed data. A $p<0.05$ was considered to indicate a significant difference. The data were evaluated at a 95% confidence interval. The Statistical Package for the Social Sciences (SPSS Inc.; Chicago, IL, USA) 17.0 program was used for statistical analysis.

RESULTS

Effects of Postconditioning on Skeletal Muscle Enzymes

Skeletal muscle ischemia in the compressed limb was verified by significant increases of serum CK and LDH levels in the PC group compared with the control group (p=0.014 and 0.013, respectively). The mean serum levels of CK and LDH were 6329.00±1884.65 and 1447.60±576.06 U/L, respectively, in the PC group and 1352.20±575.91 and 714.40±261.55 U/L in the control group (Figures 1 and 2). These findings confirm that ischemic postconditioning was induced in lower limb skeletal muscle, as described.

Effects of Treatments on Histopathological Changes in Brain Tissue

The hemorrhagic area was significantly less in the Infx group than in the control group (p=0.002) (Figure 3). In addition, loss of the gray matter–white matter boundary was significantly less in the Infx and Lef groups than in the control group (p=0.002 for both comparisons) (Figure 4). There were nonsignificant decreases in macrophages, coagulation necrosis, interstitial edema, neutrophilic infiltration in the area surrounding necrosis, and small red neurons affected by ischemia in the Infx and Lef groups compared with the control group.

Figure 3. a, b. Appearance of hemorrhagic areas in cerebral tissue after global cerebral ischemia reperfusion
*Significant decrease in the infliximab (Infx) group (a) compared with the control group (b) (p=0.002)

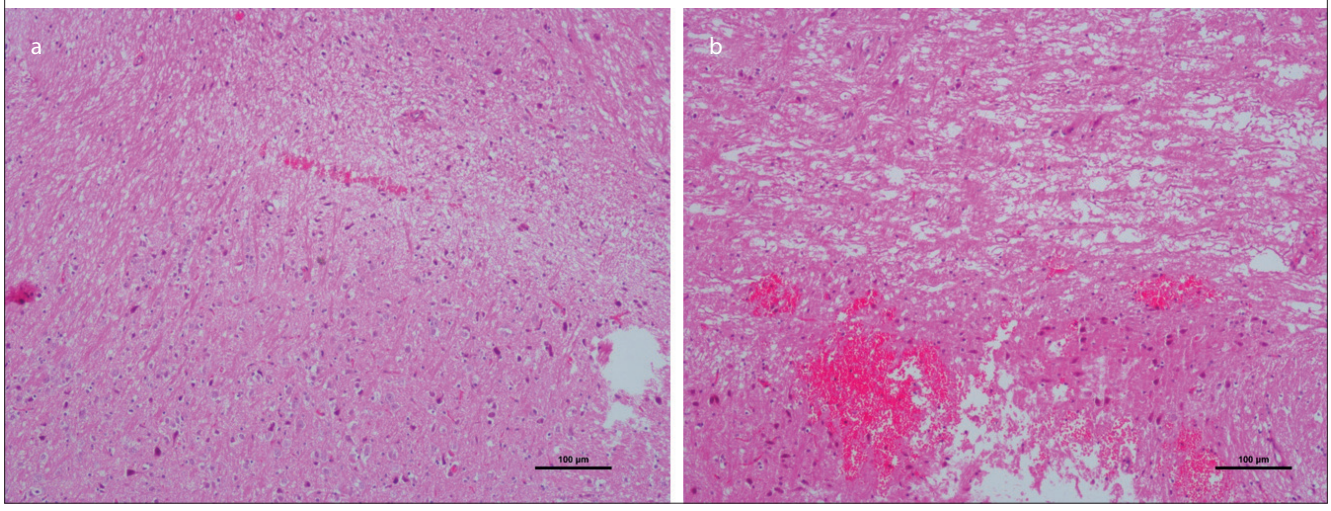


Figure 4. a, b. Loss of the gray matter–white matter boundary after global cerebral ischemia reperfusion
*Significant decrease in the infliximab (Infx) group (a) compared with the control group (b) (p=0.002)

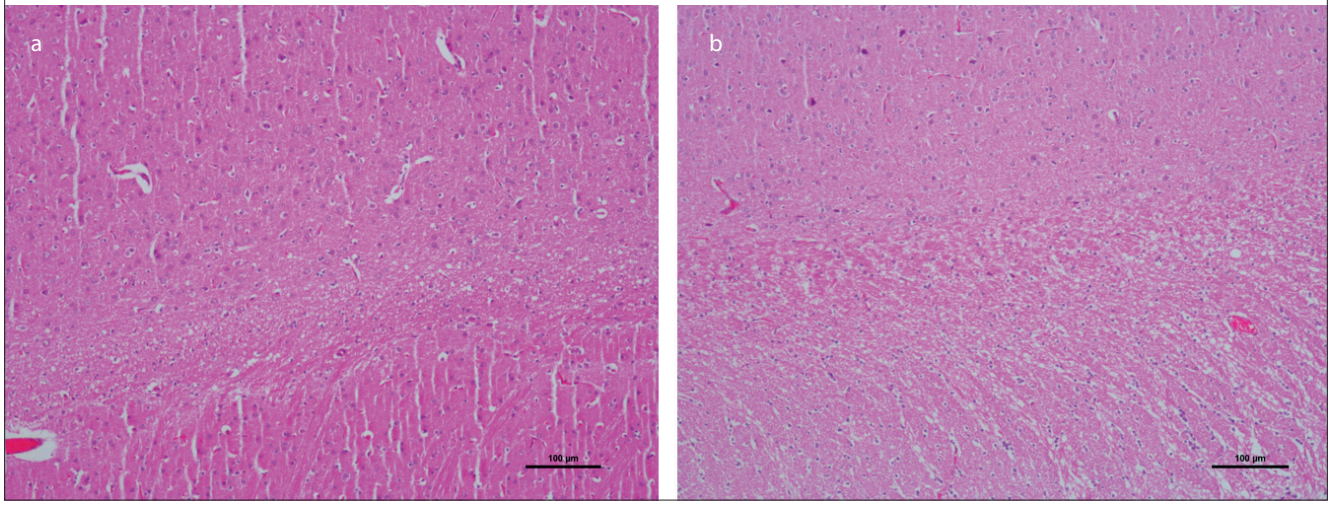


Table 1. Hypoxia-induced oxidant markers in the brain and serum of rats with pharmacologic (infliximab or leflunomide) and nonpharmacologic (ischemic postconditioning) treatments after global cerebral ischemia

Variable	PC	Infx	Lef	Control
Brain GSH-Px (nmol/g)	288.17±30.51	293.10±30.29*	282.93±58.37	244.92±29.12
Serum GSH-Px (nmol/mL)	56.27±9.10**	72.54±5.33	66.45±10.67**	75.12±3.13
Brain MDA (nmol/g)	14.64±2.78***	13.07±2.50***	6.49±1.24	6.85±0.94
Serum MDA (nmol/mL)	6.73±1.07	6.78±1.06	6.39±2.17	6.69±5.36
Serum IMA (absU)	0.34±0.05	0.31±0.08	0.33±0.07	0.35±0.02
Serum CK (U/L)	6329.00±1884.65[a]	1978.00±2479.08	1540.25±509.14	1352.20±575.91
Serum LDH (U/L)	1447.60±576.06[b]	635.75±442.01	1077.75±186.72	714.40±261.55

PC: postconditioning group; Infx, infliximab group; Lef: leflunomide group; GSH-Px: glutathione peroxidase; MDA: malondialdehyde; IMA: ischemia-modified albumin; CK: creatine kinase; LDH: lactate dehydrogenase

*Significant increase in the Infx group compared with the control group (p=0.035). **Significant decrease in the PC and Lef groups compared with the control group (p=0.023). ***Significant increase in the PC and Infx groups compared with the control group (p=0.003 and 0.002). [a,b] Significant increase in the PC group compared with the control group (p=0.014 and 0.013).

Figure 5. Brain glutathione peroxidase (GSH-Px) levels after global cerebral ischemia reperfusion

*Significant increase in the infliximab (Infx) group compared with the control group (p=0.035)

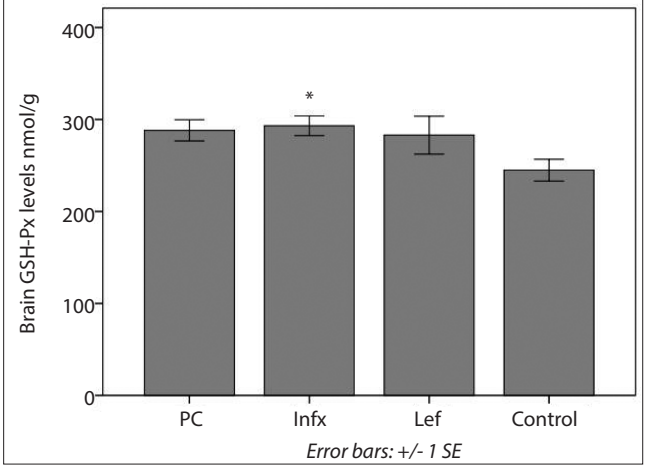


Figure 6. Serum malondialdehyde (MDA) levels after global cerebral ischemia reperfusion

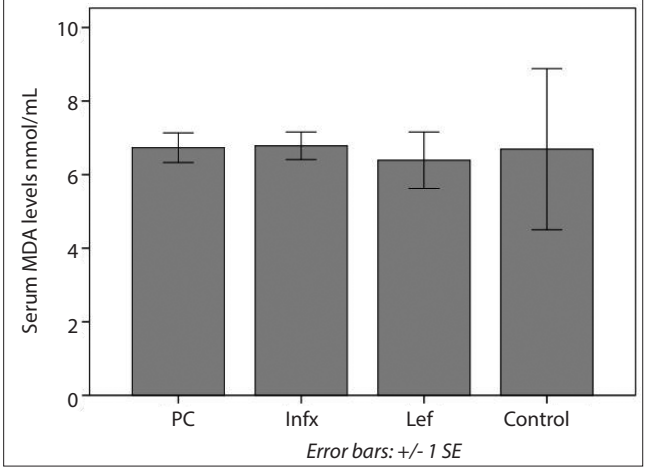
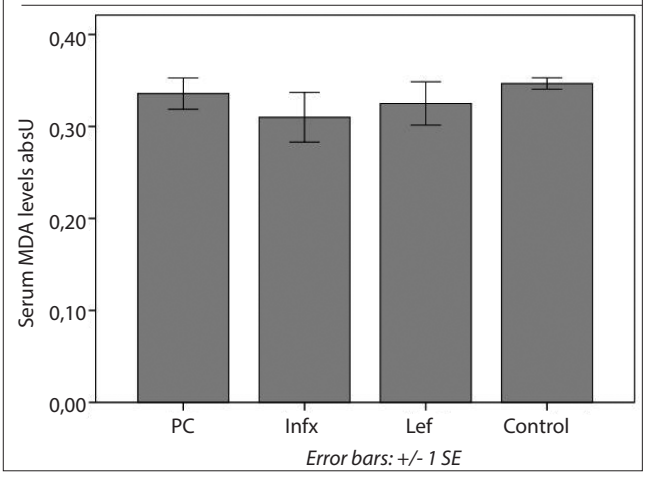


Figure 7. Serum ischemia-modified albumin (IMA) levels after global cerebral ischemia reperfusion



Effects of Treatments on Hypoxia-Induced Oxidant Markers in Brain Tissue

We evaluated the brain tissue levels of GSH-Px and MDA in the PC, Infx, and Lef groups compared with the control group. The mean level of brain GSH-Px was significantly greater in the Infx group (293.10±30.29 nmol/g) than in the control group (244.92±29.12 nmol/g) (p=0.035) (Table 1 and Figure 5). However, the mean levels of brain MDA in the PC group (14.64±2.78 nmol/g) and the Infx group (13.07±2.50 nmol/g) were significantly greater than that in the control group (6.85±0.94 nmol/g) (p=0.003 and 0.002, respectively).

Effects of Treatments on Hypoxia-Induced Oxidant Markers in the Serum

We evaluated the serum levels of GSH-Px, MDA, and IMA in the PC, Infx, and Lef groups compared with the control group. The mean serum levels of GSH-Px were 56.27±9.10 nmol/mL in the PC group, 56.73±24.49 nmol/mL in the Lef group, and 75.12±3.13 nmol/mL in the control group. These findings indicate that serum GSH-Px was significantly reduced in the PC and Lef groups

(p=0.023 for both comparisons). There were no significant differences in the serum levels of MDA or IMA between the treatment groups and the control group (Figures 6, 7).

DISCUSSION

This study compared the effects of ischemic postconditioning on hypoperfused cerebral tissue with the effects of infliximab and leflunomide. First, we demonstrated that postconditioning resulted in significant increases in the serum levels of CK and LDH, verifying the destruction of skeletal muscle in the compressed limb in the ischemic postconditioning model. Second, we found that immunosuppression by infliximab and leflunomide, but not ischemic postconditioning, may lead to significant induction of antioxidant activity in serum and ischemic brain tissue after vascular occlusion.

There are many factors in the pathogenesis of stroke caused by cerebral ischemia. Studies have shown that cytokines may have a role in this process. In cerebral ischemia, the release of various local and systemic cytokines, in particular TNF-α, is induced. TNF-α may cause either the protection or damage of nerve cells following ischemia. In the inflammation process, TNF-α is involved in the release of chemoattractant cytokines, up-regulation of endothelial adhesion molecules, migration of leukocytes, and induction of the endothelium to the prothrombotic stage (8). TNF-α is involved in ischemic injury, and blockade of endogenous TNF-α is claimed to be protective against neuronal injury. Studies showed that high serum levels of TNF-α persisted for 7 days after stroke and were correlated with the severity of the cerebral infarction area (9, 10). We found that the use of the TNF-*α* inhibitor infliximab decreased the area of the hemorrhagic region in the rat brain after global cerebral ischemia. The number of red blood cells in the extravascular area was less in rats treated with infliximab when the agent was administered immediately after and 6 h after the induction of ischemia, compared with untreated rats. Infliximab was administered at two different times because of changes in the dynamics of TNF-*α* and its receptors, depending on the time of its administration (8). Similarly, we showed that loss of the distinction between gray and white matter of the brain after stroke was significantly decreased in the Infx group.

TNF-α also acts as a potent inducer of reactive oxygen species through impairment of mitochondrial biogenesis and activation of NADPH oxidase (11). In previous studies, treatment of ischemia with infliximab increased *superoxide dismutase (*SOD) activity and decreased GSH levels in spinal cord tissue (12). In a rat model of the ischemic kidney, SOD and GSH levels were significantly higher in the infliximab groups than in the ischemia group (13). In our study, the TNF-α inhibitor infliximab increased GSH-Px levels in brain tissue during stroke.

Leflunomide, an isoxazole derivative and pyrimidine analog, suppresses proinflammatory cytokines as a main target in anti-inflammation and immune regulation. Our findings indicated that loss of the distinction between the gray and white matter of the brain was significantly decreased in the Lef group, in association with and likely mediated by suppression of proinflammatory cytokines.

Several studies indicated that leflunomide increased the expression of antioxidant enzymes such as NAD(P)H quinone dehydrogenase 1 (NQO1), catalase, and SOD. These results supported the hypothesis that leflunomide decreases oxidative stress in human pulmonary arterial endothelial cells via SOD2- and catalase-dependent, but aryl hydrocarbon receptor- and NQO1-independent, mechanisms (14, 15). However, we found that leflunomide treatment did not improve the serum or brain antioxidant levels of rats that received 10 mg/kg of leflunomide immediately after and 6 h after stroke. This short period may not be sufficient for improvement of the redox state. The mechanisms by which this drug induces NQO1 in vivo are unknown. Leflunomide was reported to cause significant induction of the expression of pulmonary CYP1A1 and NQO1 in neonatal mice. Interestingly, the dose at which leflunomide increased NQO1 was significantly higher than that required to induce CYP1A1 enzyme (15). We may find improvement in the antioxidant state if we explore the levels of leflunomide in the later period, since leflunomide has a long half-life.

Preconditioning is a protective condition in which subinjury stress can lead to protection against the effects of stroke. Ischemic preconditioning acts in a variety of tissues, including the brain. Hypoxic preconditioning increases disruption of the blood–brain barrier through a vascular endothelial growth factor (VEGF)-related pathway and suggests the possibility of aggravation of brain edema by hypoxic preconditioning in the early stages of cerebral ischemia (16). Preconditioning is a form of defense that stimulates endogenous protective mechanisms. Many pharmacologic agents, such as metabolic inhibitors, volatile anesthetics, K^+ATP channel activators, inflammatory mediators, and some natural dietary compounds, are defined as preconditioning stimuli. Some nonpharmacologic conditions, such as repetitive hyperbaric oxygen, normobaric hyperoxia, hypo- and hyperthermia, depression, acupuncture, and exercise, may also trigger cerebral preconditioning that reduces ischemic brain damage (5). In animals, preconditioning has been well documented as an effective mechanism for protecting the brain from injury. However, there are concerns over how these results will translate to humans with chronic diseases.

Ischemic postconditioning consists of a series of rapid, intermittent interruptions of blood flow during reperfusion, and it stimulates the same protective mechanisms as preconditioning. Since most cerebral ischemic events occur unpredictably, postconditioning could provide more therapeutic benefits than preconditioning. Up-regulation of the expression of antiapoptotic factors and neurotropins and modulation of the activities of several protein kinases and transcription factors, such as hypoxia-inducible factor-1 (HIF-1), are considered the most important aspects of the neuroprotective potential of postconditioning (17). Multiple mechanisms have been suggested to contribute to the neuroprotective mechanisms of ischemic postconditioning, such as regulation of synaptic signaling, reduction in oxidative stress and inflammation, maintenance of mitochondrial integrity, decrease in endoplasmic reticulum stress, activation of the phosphoinositide 3-kinase/Akt pathway, inhibition of apoptosis, and protection of the neurovascular unit (18). In the study of Chu et al. (19),

ischemic postconditioning, similar to preconditioning, improved the functions and transmembrane potential of mitochondria in rats after ischemia/reperfusion. They found that ischemic postconditioning reduced the release of cytochrome C by inhibiting the decrease in the transmembrane potential of mitochondria, thus reducing the occurrence of apoptosis. Schewe et al. (20) reported that reduced efflux of thromboxane B2 was a possible mechanism for the effect of ischemic postconditioning. Sun et al. (18) claimed that activation of autophagy was involved in the beneficial effects of ischemic postconditioning by reducing infarct volume.

In our study, however, ischemic postconditioning had no significant positive effects on the recovery of neurons in the penumbra region in the early period in rats with ischemic stroke. Our findings may be consistent with the presence of dysfunctional autophagic flux during reperfusion, possibly due to impaired autosomal degradation. Gao et al. (21) found that rapamycine, an inducer of autophagy, attenuated the effects of ischemic postconditioning. In support of this finding, in biochemical analysis we found that MDA levels in brain tissue were increased and serum GSH-Px was reduced in the PC group compared with the control group. Similar to our findings, in a study of an acute ischemia–reperfusion kidney injury, ischemic preconditioning and ischemic postconditioning, together or separately, were unable to preserve kidney function and did not exert a protective effect against tubular cell injury (22). These findings suggest that despite being essential for the survival of ischemic tissue, postconditioning may lead to additional cellular injuries. This dual effect of post-conditioning may depend on the lengths of the postconditioning cycles in the experimental protocols, as we induced ischemic postconditioning in skeletal muscle for 180 min, and subsequently reperfusion was allowed for 120 min. Shorter- and intermediate-length cycles of postconditioning were reported to enhance the mucosal microcirculation and redox state. Furthermore, milder histopathologic lesions and lower concentrations of serum proinflammatory cytokines were observed in previous studies with shorter- and intermediate-length postconditioning cycles (23). Li et al. (24) studied brief ischemic postconditioning and found that a brief episode of global brain ischemia (3 min) conducted at 1, 3, or 7 days provided neuroprotection against amyloid-β peptide neurotoxicity. The underlying mechanism was reported to be up-regulation of NMDA receptor signaling and down-regulation of mixed lineage kinase-3 (MLK3)–mitogen-activated protein kinase signal events. Most studies so far have been related to models of so-called rapid ischemic postconditioning, when reperfusion interruption is conducted in early stages (seconds and minutes) after ischemia; however, in addition to early ischemic postconditioning, "delayed" and distant ischemic postconditioning of the brain are recognized (17). Hence, the efficacy of ischemic postconditioning may be due to the lengths of the postconditioning cycles.

CONCLUSION

Although ischemic postconditioning is a technique that was found to be effective in previous experimental studies, the lengths of the postconditioning cycles are important for its efficacy. Postconditioning is not a part of current treatment protocols, as there is not enough evidence to suggest its routine use to treat ischemic injury. Furthermore, immunosuppression by infliximab and leflunomide results in significant reduction in brain ischemia after vascular occlusion. Larger studies are required to demonstrate the positive effects of postconditioning on clinical outcomes.

Ethics Committee Approval: Ethics committee approval was received for this study from the local Animal Experimentation Ethics Committee of Kahramanmaraş Sütçü İmam University (file no. 2018/07/02, approval date 10.04.2018).

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

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

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