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Pre-Treatment with Metformin in Comparison with Post-Treatment Reduces Cerebral Ischemia Reperfusion Induced Injuries in Rats

Mojtaba Karimipour*[1], *Sara Shojaei Zarghani*[2], *Majid Mohajer Milani*[3], *Hamid Soraya*[2,3]

[1]*Neurophysiology Research Center, Department of Anatomy, Urmia University of Medical Sciences, Urmia, Iran*
[2]*Cellular and Molecular Research Center, Urmia University of Medical Sciences, Urmia, Iran*
[3]*Department of Pharmacology, Urmia University of Medical Sciences, Urmia, Iran*

***Corresponding author:** *Hamid Soraya*
Address: Department of Pharmacology,
Faculty of Pharmacy, Urmia University of Medical Sciences, Urmia, Iran.
e-mail: hamid_soraya2000@yahoo.com; soraya.h@umsu.ac.ir

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ABSTRACT

Objective: To explore the effects of pre *versus* post ischemic treatment with metformin after global cerebral ischemia in rats.
Methods: Male Wister rats underwent forebrain ischemia by bilateral common carotid artery occlusion for 17 min. Metformin (200 mg/kg) or vehicle was given orally by gavage for 7-14 days. Rats were divided into: control, metformin pre-treatment, metformin post-treatment and metformin pre and post continuous treatment groups. Cerebral infarct size, histopathology, myeloperoxidase and serum malondialdehyde were measured 7 days after ischemia.
Results: Histopathological analysis showed that metformin pre-treatment significantly decreased leukocyte infiltration, myeloperoxidase activity and also malondialdehyde level. Metformin pre-treatment and metformin post-treatment reduced infarct size compared with the control group, but it was not significant in the pre and post continuous treatment group.
Conclusion: Our findings suggest that pre-treatment with metformin in comparison with post-treatment in experimental stroke can reduce the extent of brain damage and is more neuroprotective at least in part by inhibiting oxidative stress and inflammation.

Keywords: Global cerebral ischemia; Metformin; Oxidative stress; Inflammation.

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Introduction

Stroke is one of the common pathological conditions that threats public health worldwide and is responsible for approximately 9% of total deaths each year [1-4]. Based on World Health Organization data, 15 million people suffer from stroke. Stroke can be divided into ischemic and

hemorrhagic types [5, 6].Ischemic stroke causes about 83% of all strokes and is the main leading cause of death and disability worldwide [7]. Global cerebral ischemia model is one of the experimental models that typically occur following cardiac arrest, shock, asphyxia and complex cardiac surgery [8]. Oxidative stress plays a major role in the pathogenesis of ischemia/ reperfusion (IR) injury in many organs including brain [9]. The brain contains high amounts of polyunsaturated lipids and high rate of oxidative metabolic activity. Therefore, it is one of the most important targets for free radical attacks that results in oxidative damage including lipid peroxidation, protein oxidation and DNA damage, which can lead to cell death [10, 11]. There is no proven drug or pharmacologic agent to reduce neuronal injury after global cerebral ischemia in humans [12, 13]. And now many scientists are investigating neuroprotective effects of various drugs to be used in humans. Metformin is introduced for the treatment of types 2 diabetes mellitus since 1960s. This drug is effective in treating diabetes by inhibition of intestinal glucose absorption and reduction of gluconeogenesis in the liver [14].

In recent years, many studies have shown that the effect of metformin are not entirely attributed to its antihyperglycemic effects and has other useful properties including favorable cardiovascular and cerebrovascular effects [15, 16]. Other many previous studies have shown that metformin mitigated IR induced brain damage and has potent neuroprotective effects [17-19]. Metformin decreases blood-brain barrier disruption in mice after middle cerebral stroke [20-22]. Furthermore, it has been also reported that long-term treatment with metformin prior to stroke is neuroprotective [23]. A study demonstrated that metformin possesses antioxidant properties and improves the oxidative stress induced by global cerebral ischemia in rats [9]. Metformin also is able to act against apoptotic cell death in primary cortical neurons of cultured rats [24].

As mentioned above, pre-treatment with metformin alleviates detrimental effects of ischemia on brain morphology and functions. Little data available about neuroprotective of post-treatment of metformin following cerebral ischemia and also there is no report to compare the therapeutic effects of metformin pre and post treatment on cerebral ischemia. Therefore, the purpose of the present study was to investigate the difference in effects of metformin pre and post treatment in a rat model of global cerebral ischemia.

Materials and Methods

Animals

Healthy adult male Wistar rats weighting 230±20g were used in this study. Rats were housed in Animal House of Urmia University of Medical Sciences at a controlled ambient temperature of 23±2 ºC, with food and water available ad libitum under a 12-h light/12-h dark cycle. The present study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of Urmia University of Medical Sciences.

Chemical Reagents

Metformin was a generous gift from MahbanChemi Pharmaceutical Inc. (Tehran, Iran). Ketamine and Xylazine was from Alfasan company (The Netherlands). The other reagents were of a commercial analytical grade.

Experimental Design

Forty adult male rats were used in the whole experiment. The rats were randomly divided into four groups and were gavaged orally with metformin (200mg/kg/day) or saline. Rats in group 1 (control) underwent on bilateral common carotid ligation for induction of cerebral ischemia and received saline. Rats in group 2 (Pre) received metformin (200 mg/kg/day) for 7 days before cerebral ischemia and then received saline for next 7 days. Rats in group 3 (Post), 7 days before cerebral ischemia received saline and then treated with metformin for next 7 days. Rats in group 4 (Pre-Post) within 7 days before and 7 days after ischemia treated with metformin. Grouping of the animals and time and dose of treatment were based on previous studies [25, 26].

Surgery Procedure

The animals were anesthetized by aninjection of a mixture of ketamine (60 mg/kg, Alfasan, The Netherlands), xylazine (10 mg/kg), and then, placed in a supine position and a ventral midline skin incision was made from the lower mandible posterior to the sternum (~3 cm) to expose the right and left common carotid artery. Then, rats were subjected to 17 min of bilateral common carotid occlusion (with special clamp) [27].

Histopathological Examination

For histopathological examination, 24 h after the last treatment the rats were anesthetized and after obtaining blood and sacrificing with ketamine overdose brains were quickly removed and fixed in 10% formalin. After embedding in paraffin, 5 µm sections were stained with hematoxylin and eosin (H&E). The profile and degree of leukocyte infiltration into the brain were evaluated in each section of the brain tissue using a morphometric point-counting procedure. Two trained person were quantified cerebral damages by scoring and given a number for each changes as follows: 1, 2, 3, and 4 for low, moderate (focal cerebral damage or small multifocal degeneration with slight degree neutrophil infiltration), high (extensive degeneration and/or diffuse neutrophil infiltration), and intensive (necrosis with diffuse neutrophil infiltration) pathological changes, respectively and then photographed under light microscopy [28, 29].

Myeloperoxidase Assay

Myeloperoxidase (MPO) activity assay was performed as described previously [30, 31] with minor modifications. Brain tissues were homogenized (IKA Hemogenizer, Staufen, Germany) in a solution containing 0.5% hexa-decyltrimethyl-ammonium bromide (HTAB) dissolved in 50 mM potassium phosphate buffer (pH 6). The samples were then centrifuged at 4500 rpm for 30 min at 4 °C. Then, samples were sonicated for 10 s, freeze-thawed three times with sonication between cycles. Suspensions were then centrifuged at 4500 rpm for 30 min. An aliquot of the supernatant (0.1 ml) or standard (Sigma, Germany) was mixed with 2.9 ml solution of 50 mM potassium phosphate buffer at pH 6 containing0.167 mg/ml of O-dianisidine hydrochloride and 0.0005% H_2O_2. After 5 min, the reaction was stopped with 0.1 ml of 1.2 M hydrochloric acid. The rate of change in absorbance at 460 nm was measured using a spectrophotometer (Cecil 9000, Cambridge, UK). MPO activity was expressed in milli unit (mU) per gram weight of wet tissue.

Infarct Size Measurement

On day 15th, brain samples were quickly removed and kept at −20°C for 15 min to make a proper section. Then slices of 2 mm thickness were cut and completely immersed in a 1% solution of 2,3,5 Triphenyltetrazolium chloride (TTC) for 30 min at a temperature of 37°C in a darkroom. After staining, brain slices were fixed with 4% paraformaldehyde. The stained sections were digitally photographed and measured using Image j software [32]. Normal areas stain red whereas infarcted areas remain pale.

Determination of Malondialdehyde

Malondialdehyde (MDA) was measured as a marker of oxidative stress in serum after cerebral ischemia/ reperfusion using a method as previously described [33].

Results

Histopathological of Brain

Histological sections revealed the infiltration of leukocyte in the brain tissue of control group (Figure 1). It was found that metformin pre-treatment significantly ($p<0.05$) reduced leukocyte infiltration after global cerebral ischemia as shown in Figure 2.

Myeloperoxidase Activity

Cerebral ischemia resulted in an increase in leukocyte infiltration into the brain tissue, as measured by an increase in MPO activity in the brain (Figure 3). The pre-treatment of the rats with metformin, which was started 7 days before ischemia, was found to reduce the enzyme activity in the brain tissue from 267±22mU/g wet tissue in control group to 189±20mU/g ($p<0.05$) in metformin pre-treated group (Figure 3).

Infarct Size

The effect of metformin treatment on brain infarct size was measured by TTC staining. As shown in Figure 4A, there was a clear difference between viable tissue (a deep red) and injured tissue (pale colored). Metformin pre-treatment and metformin post-treatment significantly reduced infarct size as compared to control group (Figure 4B).

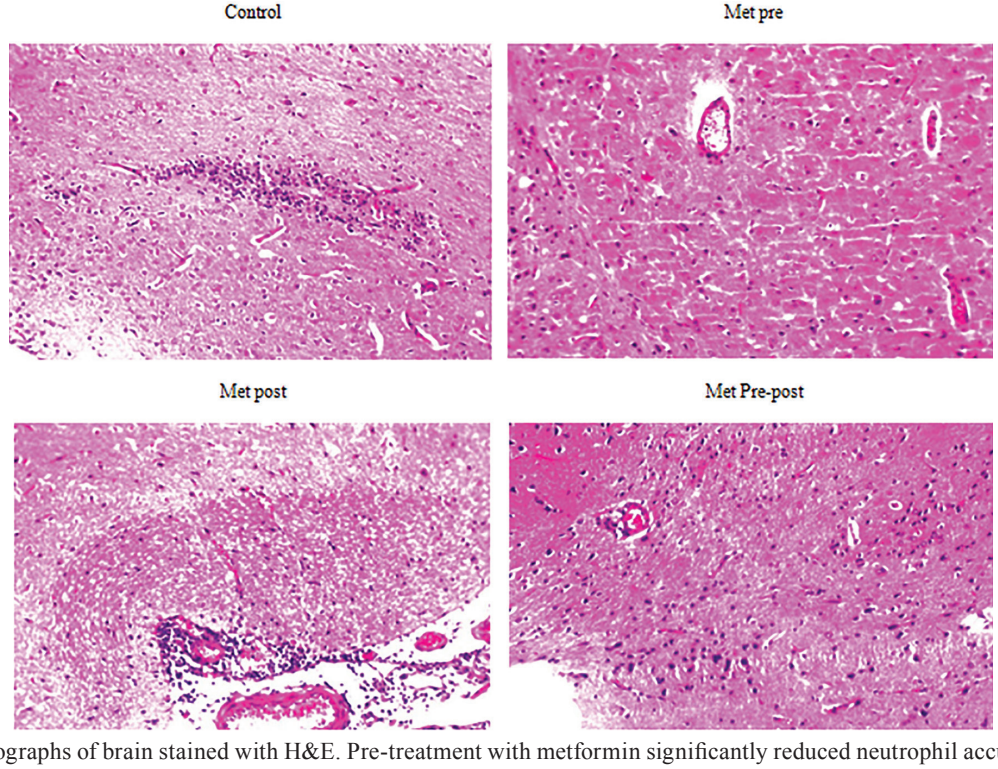

Fig. 1. Photomicrographs of brain stained with H&E. Pre-treatment with metformin significantly reduced neutrophil accumulation. Met pre: Metformin pre-treatment; Met post: Metformin post-treatment; Met pre-post: Metformin pre and post treatrment. Arrows indicate leukocyte infiltration.

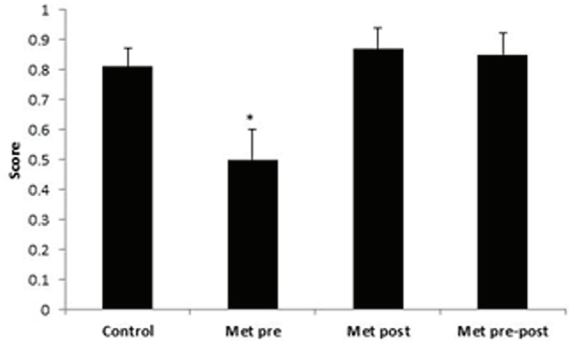

Fig. 2. Grading of leukocyte infiltration in the brain tissues. MetPre: metformin pre-treatment; Met Post: metformin post-treatment; Met Pre-Post: metformin pre and post treatment. *p<0.05 *vs* control group.

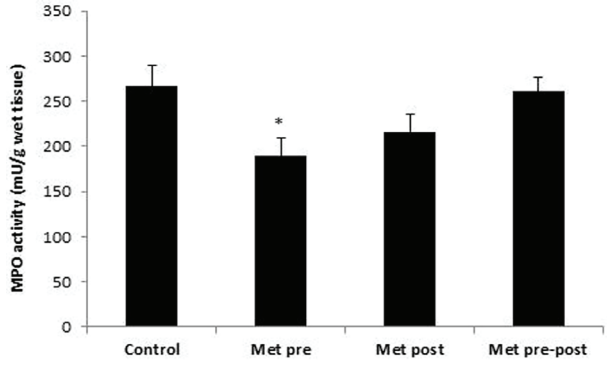

Fig. 3. The effect of metformin on brain MPO activity after cerebral ischemia. Met Pre: metformin pre-treatment; Met Post: metformin post-treatment; Met Pre-Post: metformin pre and post treatment. * p<0.05 *vs* control group.

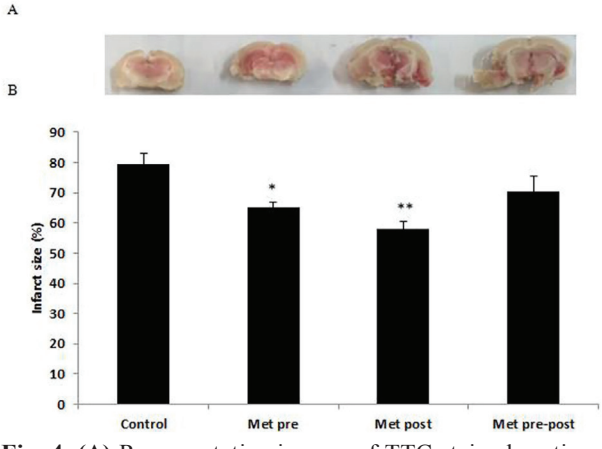

Fig. 4. (A) Representative images of TTC stained sections of brain after brain ischemia **(B)** Bar graph shows analysis of infarct size. Metformin pre-treatment and post-treatment significantly reduced infarct size. Met Pre: metformin pre-treatment; Met Post: metformin post-treatment; Met Pre-Post: metformin pre and post treatment. *P<0.05, **P<0.01 *vs* control.

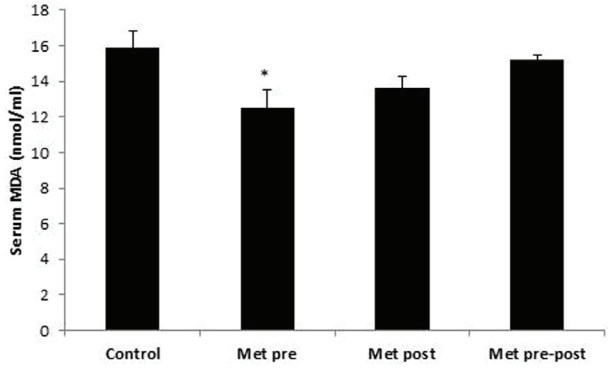

Fig. 5. Levels of serum MDA after brain ischemia. Met Pre: metformin pre-treatment; Met Post: metformin post-treatment; Met Pre-Post: metformin pre and post treatment. *P<0.05 *vs* control group.

Malondialdehyde

It was found that metformin pre-treatment significantly (p<0.05) reduced serum MDA level after cerebral ischemia, as shown in Figure 5.

Discussion

In the present study, we showed that pre-ischemic administration of metformin reduced cerebral infarct size, MPO activity, leukocyte accumulation and also serum MDA level (Table 1). Clamping of bilateral carotid arteries in rats is a common model of incomplete global cerebral ischemia that induces partial ischemia without affecting the circle of Willis but results in a 50% decrease in cerebral blood flow [34]. An important cause of death following cardiopulmonary resuscitation is global cerebral ischemia after cardiocirculatory arrest [35]. There is no drug which has been proven to decrease brain injury after global cerebral ischemia in humans [12, 13]. However, if an agent could decrease the cerebral ischemic size or amend neurological signs after global cerebral ischemia, it would be effective for patients at risk of cerebral ischemia injury [36].

Metformin is a drug widely used for the treatment of type 2 diabetes mellitus, but recently, it has been well documented that metformin has neuroprotective role on cerebral IR. For example, Liu *et al*., [25] showed that metformin could promote focal

Table 1. Summary of results along with comparison between groups.

	Control	Pre-treatment	Post-treatment	Pre-post treatment	*p* value
Neutrophil infiltration	0.81±0.06	0.5±0.1[a]	0.87±0.07	0.87±0.07	0.012[a]
MPO activity	267±22	189±20[a]	216±20	261±16	0.033[a]
MDA level	15.8±1	12.5±1[a]	13.6±0.7	15.2±0.3	0.038[a]
Infarct size	79.3±3.7	65±1.7[a]	58±2.5[b]	70.3±5	0.038[a] 0.002[b]

[a]Treatment groups compared with control group using one way ANOVA with Student-Newman-Keuls post hoc test; [b]Data are expressed as mean±sem.

angiogenesis and neurogenesis and reduce injury in mice after middle cerebral artery occlusion. Ashabi *et al*., [37] found that metformin pre-treatment is able to improve stroke signs in the rats with global cerebral ischemia. In another study, it has been reported that treatment with metformin 24h after stroke, decreases brain atrophy [22]. Results of our study provide evidence that neuroprotective effect of metformin in pre-ischemic oral administration is better in compared with post and continuous (pre and post) administration. To our knowledge, there is no report to compare the neuroprotective effect of metformin in different time of oral administration (pre, post and continuous pre-post) on global cerebral ischemia.

Regarding to pathophysiological of brain ischemia, several mechanisms are involved in inducing cell damage including, inflammation, apoptosis and oxidative stress. [38]. Neuroprotective effects of metformin in cerebral ischemia are mediated through multiple mechanisms in which several different pathways act closely with each other. There are reports proposed the protective role of metformin via activation the AMP activated protein kinase (AMPK) in neuronal, cardiac and renal cells under ischemic condition [39, 40]. AMPK has a pivotal role in regulating energy homeostasis and cell survival [41]. The role of AMPK in brain diseases such as ischemia, Alzheimer and Huntington`s is well known [42-44]. In previous studies, it has been documented that metformin through phosphorylation of AMPK causes downstream signaling pathways in neurons during oxidative stress and neurodegeneration [37, 45]. Other reports demonstrated that AMPK is activated by metformin administration which in turn activates some pathway including, mitochondrial biogenesis, antioxidant enzymes and nuclear factor erythroid 2 related factor (Nrf2) [37, 42].

In the current study, the MDA level, as an oxidative stress marker, was reduced significantly in the metformin pre-treatment group. This result is in accordance with the study indicating that metformin inhibits the production of free radicals [9]. Furthermore, it has been also reported that metformin reduces ROS levels by inducing antioxidant thioredoxin expression via activation of the AMPK [46]. Another mechanism that metformin exerts its antioxidant property and inhibits inflammatory responses is activation of nuclear factor erythroid 2 related factor (Nrf2) pathway through induction of AMPK following transient global cerebral ischemia model [42]. We demonstrated that pre-treatment with metformin for 7 days before cerebral ischemia, significantly decreased infarct size. This finding is consistent with other studies which used the chronic metformin administration [23] although acute pre-treatment (3 days) has been shown to increase injury in ischemic stroke [47].

Inflammation is one of important pathologic factor involved in brain damage in cerebral ischemia [48]. Nuclear factor kappa B (NF-KB) as a transcriptional regulator factor in inflammation is activated during brain ischemia and leads to production of pro-inflammatory cytokines such as tumor necrosis factor (TNF-α),interloukin (IL)-1β, IL6 and inducible nitric oxide synthase (iNOS) [49, 50].

Pervious experimental studies in rodents showed that inhibition of NF-KB could decreased inflammation and improve brain ischemia outcome [51, 52].

It has been documented that pre-treatment with metformin is able to decrease inflammation through suppression of NF-KB during acute phase of ischemic stroke [53].

To demonstrate the effects of metformin on neutrophil infiltration, we performed MPO assay 7 days after cerebral ischemia. Results showed that metformin pre-treatment reduced MPO activity. The mechanism by which metformin decreases an inflammatory response after cerebral ischemia is not fully understood. Metformin decreased ILβ, IL6 and TNFα expression in mice 3 days following middle cerebral artery occlusion [20]. Previous reports have shown that intercellular adhesion molecule1 (ICAM1) expression increases during reperfusion and leads to an increase in neutrophil adhesion to ECs and enhances their infiltration [40] and anti-ICAM1 decreases infarct size [54]. Liu *et al*., [20] showed that metformin reduced ICAM1 via the AMPK signaling pathway both in vivo and in vitro and this reduction *in vivo* was accompanied by alleviated leukocyte infiltration and reduced infarct size in mice after middle cerebral artery occlusion. Thus, it has been concluded that metformin exerts its neuroprotective effects following cerebral ischemia partly through decreased ICAM1 expression.

Like other studies, our project has some limitations. For example: the pro-inflammatory cytokines levels, activity of antioxidant enzymes and AMPK expression were not measured. Evaluating each of them could help in understanding the protective mechanisms of metformin against cerebral ischemia. In our study the animals were euthanized 7 days after brain ischemia and long-term effects of metformin administration were not evaluated.

In conclusion our study showed that metformin pre-treatment in comparison to post and pre-post treatment, reduced significantly cerebral ischemia induced injuries at least in part through its anti-inflammatory and antioxidant effects.

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Conflicts of Interest: None declared.

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