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Article

Effects of pre-treatment with metoprolol and diltiazem on cerebral ischemia/reperfusion-induced injuries

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Stroke is one of the most important health concerns worldwide. Calcium ions accumulation in the nerve cells and increase in the catecholamines level of the brain following cerebral ischemia/reperfusion (I/R) are accompanied by damaging effects. Therefore, the present study aimed to evaluate the effects of diltiazem, as a calcium channel blocker, and metoprolol, as a β-adrenoceptors antagonist, on I/R injury. In this study, 30 male Wistar rats were divided into control, I/R, metoprolol, diltiazem, and metoprolol plus diltiazem groups (n=6 in each). Metoprolol (1 mg/kg/day) and diltiazem (5 mg/kg/day) were injected intraperitoneally (i.p.) for 7 days before I/R induction. On day 8, the animals underwent ischemia by bilateral common carotid arteries occlusion for 20 min. Histopathological analysis showed a significant reduction in leukocyte infiltration in diltiazem, metoprolol, and diltiazem plus metoprolol treated rats compared with the I/R group ($P<0.05$, $P<0.01$, $P<0.01$, respectively). In addition, in all treated groups, myeloperoxidase activity and malondialdehyde levels in the brain tissue significantly declined compared with the I/R group ($P<0.001$). Furthermore, pre-treatment with diltiazem and metoprolol alone or in co-administration remarkably reduced infarct size following I/R ($P<0.001$). Overall, the results indicate the considerable neuroprotective effects of metoprolol and diltiazem in cerebral I/R.

Keywords: Metoprolol. Diltiazem. Brain ischemia., Infarct size. Oxidative stress.

INTRODUCTION

Today, stroke is a major public health problem and among the most common life-threatening neurological diseases, such that it is the third leading cause of death worldwide after cardiovascular disease and cancer (Donkor, 2018). Stroke as a sudden focal neurological deficit due to cerebrovascular disease can be divided into ischemic and hemorrhagic types. About 83% of brain strokes are ischemic strokes and are the main leading cause of disability in the world (Chandra *et al.*, 2017). Some studies have shown that global cerebral ischemia in rats significantly increases cerebral infarct size. Infarct size is a factor for measuring necrotic areas of tissue. In this respect, neuroprotective effects may attribute to a reduction in the brain infarction size (Zhang *et al.*, 2014). During ischemia, the amount of oxygen and adenosine 5'-triphosphate (ATP) in the brain decreases. Consequently, acidosis and cell damage occur in the ischemic area due to mitochondrial function impairment caused by free radicals and lactate accumulation. In this sense, reducing antioxidant activity and capacity induces serious damage to cellular components such as lipids called lipid peroxidation, proteins, and nucleic acids, ultimately leading to cell death (Chan, 2001). Reperfusion-induced injury results from an inflammatory function of the injured tissue, whereby re-establishing the blood flow, neutrophils release inflammatory factors such as interleukin-1 beta and free radicals in the affected tissue (Nour, Scalzo, Liebeskind, 2013). Oxidative stress plays a pivotal role in the pathogenesis of neurodegenerative and neurological diseases such as Alzheimer's disease,

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Parkinson's disease, trauma, and stroke (Chen, Guo, Kong, 2012).

Calcium channels are found in the different parts of the central nervous system (CNS), such as the cerebral cortex, hippocampus, cerebellum, and spinal cord (Cheong, Shin, 2013). Calcium channel blockers (CCBs) inhibit calcium ion flow through volumetric calcium channels. Diltiazem is a CCB that mainly affects L-type calcium channels (Mieth *et al*., 2013). This drug is effective in treating angina, hypertension, and atrial fibrillation by inhibiting extracellular calcium ion influx and cardiac and vascular smooth muscle contraction (Frishman, 2009). In several animal models, CCBs have demonstrated protective effects in the nervous tissues (Su *et al*., 2010). A study showed that diltiazem possesses antioxidant properties and prevents dementia-induced oxidative stress (Rani *et al*., 2015). Research has also demonstrated the anti-ischemic activity of calcium antagonists in the brain (Tomassoni *et al.,* 2008).

β-adrenoceptors are present at almost every mammalian cell surface. There are three subgroups of β receptors called β_1, β_2, and β_3. These adrenoceptors are widely distributed in the whole of CNS, like the neocortex, thalamus, cerebellum, hypothalamus, medulla, and brain stem (Hagena, Hansen, Manahan-Vaughan, 2016). Metoprolol, a selective β_1 adrenergic receptors blocker, is commonly used for the treatment of blood pressure, angina, myocardial infarction (MI) prevention, heart failure, migraine prophylaxis, and symptomatic treatment of acute anxiety and phobia by blocking response to β_1 adrenergic stimulation (Tucker, Sankar, Theetha Kariyanna, 2021). Some studies have reported anti-inflammatory and anti-atherosclerotic effects of metoprolol (Ulleryd *et al*., 2014). It has been shown that metoprolol can lower oxidative stress and myocardial ischemic injury (Bao *et al*., 2015). A study has also demonstrated the neuroprotective effect of β_1 antagonists on ischemia/reperfusion (I/R). It was concluded that cortical infarct volume was lower in rats receiving β-adrenoceptor antagonists and improved neurological outcomes (Goyagi, Nishikawa, Tobe, 2010). It has also been reported that β-adrenoceptor antagonists reduced the infiltration and degranulation of neutrophils in the myocardium in experimental studies (Gao *et al*., 2000). A study reported that β-blockers decrease mortality in acute subarachnoid hemorrhage in humans (Chang *et al*., 2016). However, there are no approved drugs to reduce brain injury in patients with global cerebral ischemia. In the present study, we tried to clarify whether prophylactic use of diltiazem and metoprolol can lower the brain damage caused by ischemia. Hence, we aimed to highlight the protective effects of pre-treatment with diltiazem and metoprolol on global I/R induced injuries in rats.

MATERIAL AND METHODS

Ethical statement and animals

All procedures used in this study were approved by the ethics committee of the Urmia University of Medical Sciences (Ethical code: IR.UMSU.REC.1397.285), following the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication, 8th Edition, 2011; National Research Council Committee for the Update of the Guide for the and Use of Laboratory, 2011). Adult male Wistar rats weighing 250±30 g (8-10 weeks old) were used in this study. The animals were obtained from the Animal House of Urmia University of Medical Sciences. Next, they were allowed free access to water and food and were kept at a controlled ambient temperature of 23±2ºC with 50±10 % relative humidity and a 12-h light/12-h dark cycle.

Chemical Reagents

Metoprolol was a generous gift from Alborz Darou Pharmaceutical Inc. (Alborz, Qazvin, Iran) and diltiazem was a generous gift from Amin Pharmaceutical Co. (Isfahan, Iran). The other reagents were of a commercial analytical grade.

Experimental protocol

The animals were randomly assigned into five groups, each with 6 rats. In group 1 (control), rats were given saline (0.5 ml) intraperitoneally (*ip*) for the entire

period of the experiment (14 days). Rats in group 2 (I/R) received saline by i.p. injection for 14 days. Also, for induction of cerebral ischemia, they underwent bilateral common carotid ligation for 20 min on day 8. Rats in group 3 were pre-treated i.p. with metoprolol (1 mg/kg/day) for 7 days before cerebral ischemia and then were received a saline injection for the next 7 days. Rats in group 4, were pre-treated i.p. with diltiazem (5 mg/kg/day) for 7 days before cerebral ischemia and then received saline for the next 7 days. Rats in group 5 received a combination of metoprolol (1 mg/kg/day) and diltiazem (5 mg/kg/day) 7 days before ischemia and then received saline for the next 7 days. The doses of metoprolol and diltiazem were chosen according to previous studies (Anjaneyulu, Chopra, 2005; Beril Gok et al., 2005).

Surgery procedure

For induction of global cerebral ischemia, the rats were anesthetized by i.p. injection of a mixture of ketamine 60 mg/kg and xylazine 10 mg/kg (Alfasan company, The Netherlands). This induction was performed through the bilateral carotid artery occlusion method. After deep anesthesia, rats were placed supine, and their hair neck was shaved and disinfected with Betadine. Then, a cut was made at the midline of the neck from the lower mandible posterior to the sternum (~3 cm) to find the right and left common carotid arteries and separate them from the vagus nerve and surrounding tissues. In this study, the common carotid arteries were occluded for 20 min, followed by careful withdrawal of clamps for reperfusion (Zamani *et al*., 2013).

Infarct Size Measurement

On day 15th, the cerebral infarct size of the rats was evaluated after sacrificing the rats with anesthesia. After opening the skull, the brain tissues were rapidly removed and frozen at -20ºC for 15 min. Then, they were sliced coronally at 2-mm intervals and incubated with 2% solution of 2,3,5-Triphenyltetrazolium chloride (TTC) in potassium phosphate buffer (pH 6) for 20 min at 37ºC in a darkroom. After staining, brain slices were fixed with 10% formaldehyde for 24 h. Finally, the stained sections were digitally photographed and measured using ImageJ software. It was found that normal areas stain red, while infarcted areas remain pale white (Kamat *et al*., 2015).

Histopathological Examination

After cerebral I/R, the pathological changes in the brain tissues following cerebral I/R were examined by fixing the samples in 10% formalin solution and preparing the paraffin-embedded blocks. Afterward, 5 µm thick sections were cut using a microtome and stained by hematoxylin and eosin (H&E). For evaluation of cerebral necrosis and leukocyte infiltration, two trained person (at least one pathologist) quantified histological changes by giving a score for each change as follows: 1, 2, 3, and 4 for low, moderate (small multifocal degeneration with slight degree leukocyte infiltration), high (extensive degeneration or diffuse leukocyte infiltration), and intensive (necrosis with diffuse leukocyte infiltration) pathological changes, respectively (Benjamin *et al*., 1989).

Myeloperoxidase Assay

Myeloperoxidase (MPO) activity of brain tissue as an index of the neutrophil count was measured as described previously. Briefly, brain tissue samples were placed in a solution containing 0.5% hexadecyl trimethyl ammonium bromide (HTAB) dissolved in 50 mM potassium phosphate buffer (pH 6). After homogenization, the samples were sonicated for 10 s and then were subjected to freeze and thaw cycles in triplicate. Suspensions were then centrifuged at 4000 rpm for 45 min, and 0.1 ml of supernatant or standard (Sigma, Germany) was mixed with 2.9 ml solution of 50 mM potassium phosphate buffer at pH 6 containing 0.167 mg/ml of O-dianisidine hydrochloride and 0.0005% H2O2. In the next step, 0.1 ml of 1.2 M hydrochloric acid was added to stop the reaction. The absorbance change rate at 400 nm was measured using a spectrophotometer (Cecil 9000, Cambridge, UK). Eventually, MPO activity was expressed in milli unit (mU) per gram weight of wet tissue (Mullane, Kraemer, Smith, 1985).

Determination of Malondialdehyde

The level of malondialdehyde (MDA) as an oxidative stress biomarker and a product of lipid peroxidation was measured after cerebral I/R in the brain tissues. Tissue samples were homogenized in 1.5% cold potassium chloride to make a 10% homogenate. The samples were then centrifuged at 3000 rpm for 15 min. About 0.25 ml of supernatant was mixed with 3 ml phosphoric acid 1% and 1 ml thiobarbituric acid and then put in the bain-marie for 45 min at 90°C. Next, samples were cooled and added 3 ml N-butanol followed by centrifugation at 3000 rpm for 15 min. Finally, the absorbance change rate of the butanol phase was measured at 532 nm using a spectrophotometer (Cecil 9000, Cambridge, UK). The results were expressed as nanomole MDA production per mg brain tissue (Mihara, Uchiyama, Fukuzawa, 1980; Olgen, Coban, 2003).

Statistical analysis

The results were expressed as mean ± standard error of the mean (S.E.M). Statistical significance was determined by one-way analysis of variance (ANOVA) with Tukey post hoc test using SPSS 16, where p-values less than 0.05 were considered statistically significant.

RESULTS

Effects of pre-treatment with metoprolol and diltiazem on infarct size after cerebral ischemia/reperfusion

TTC staining was performed to measure infarct size. It was observed that infarct size increased significantly in the I/R group to 56.6±4.2 compared to the control group ($P<0.001$). Pre-treatment with metoprolol (1 mg/kg) and diltiazem (5 mg/kg) individually and in combination, which was started 7 days before induction of cerebral ischemia/reperfusion, lowered infarct size significantly in all treated groups in comparison to the I/R group ($P<0.001$) (Figure 1).

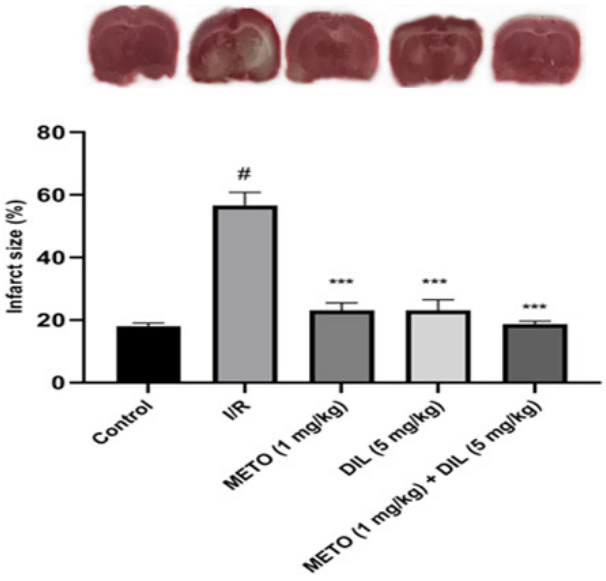

FIGURE 1 - (A) Representative images of TTC stained sections of the brain after cerebral ischemia/reperfusion; (B) The effects of pre-treatment with metoprolol (1 mg/kg/day) and diltiazem (5 mg/kg/day) alone and in combination on infarct size in cerebral ischemia-reperfusion; data are expressed as mean±S.E.M. N=6. $^{\#}$ $P<0.001$ vs. control group. *** $P<0.001$ vs. I/R group using one-way ANOVA with *Tukey* post-test. I/R: Ischemia/reperfusion; METO: Metoprolol; DIL: Diltiazem.

Effects of pre-treatment with metoprolol and diltiazem on histopathological changes after cerebral ischemia/reperfusion

Histopathological changes in different groups were evaluated using H&E staining. The rate of leukocyte accumulation in all groups was reported from 1 to 4 for low, medium, high, and extensive accumulation, respectively. No leukocyte accumulation was observed in the control group, but an intensive aggregation and heterogeneous dispersion of leukocytes was noticed in the I/R group. According to the histopathological results, pre-treatment with diltiazem ($P<0.05$) and metoprolol alone ($P<0.01$) or in combination ($P<0.01$) significantly diminished cerebral leukocyte recruitment compared to the I/R group (Figure 2).

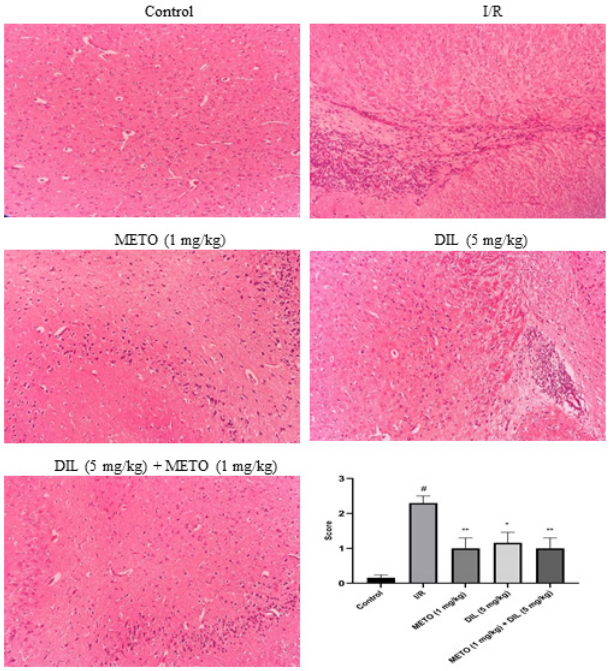

FIGURE 2 - (A) Photomicrographs of brain stained with H&E after cerebral ischemia/reperfusion; (B) Grading of histopathological changes in the rat's brain tissues. Data are expressed as mean±S.E.M. N=6. #P<0.001 vs control group. **P<0.01, *P<0.05, and **P<0.01, respectively, vs. I/R group using one-way ANOVA with *Tukey* post-test. I/R: Ischemia/reperfusion; METO: Metoprolol; DIL: Diltiazem.

Effects of pre-treatment with metoprolol and diltiazem on myeloperoxidase activity after cerebral ischemia/reperfusion

Leukocyte infiltration (especially neutrophils) into the brain tissues was also measured by MPO activity. The result showed that MPO activity significantly increased from 187.2±7.5 in the control group to 452.4±25 in the I/R group (P<0.001). Pre-treatment with metoprolol (1 mg/kg), diltiazem (5 mg/kg) alone, and in combination, which was started one week before induction of cerebral I/R, lowered MPO activity in the brain tissues to 276.3±9.8, 253.2±20.1, and 258.5±22.5 (mU/g tissue) compared to I/R group, respectively (P<0.001) (Figure 3).

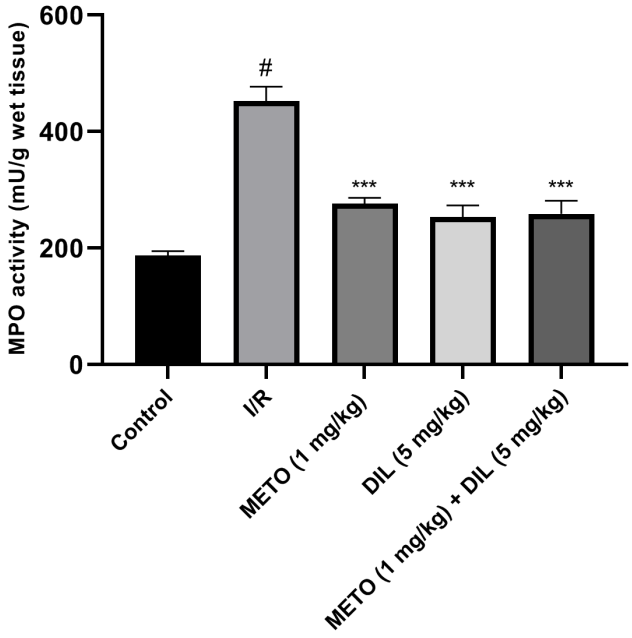

FIGURE 3 - The effects of pre-treatment with metoprolol (1 mg/kg/day) and diltiazem (5 mg/kg/day) alone and in combination on MPO activity in the brain tissue; data are expressed as mean±S.E.M. N=6. # P<0.001 vs. control group. *** P<0.001 vs. I/R group using one-way ANOVA with *Tukey* post-test. I/R: Ischemia/reperfusion; METO: Metoprolol; DIL: Diltiazem.

Effects of pre-treatment with metoprolol and diltiazem on lipid peroxidation (MDA) after cerebral ischemia/reperfusion

The lipid peroxidation was determined by measuring the MDA level in the brain tissues. Our results showed that the MDA level significantly increased from 15.1±4.2 in the control group to 54.4±5.6 in the I/R group (P<0.001). Also, pre-treatment with metoprolol (1 mg/kg), diltiazem (5 mg/kg), or in combination, which was started one week before induction of cerebral I/R, decreased MDA level to 8.2±1.1, 11±1.3, and 10.5±1.6 (nmol/mg protein) in comparison to the I/R group (p<0.001), respectively (Figure 4).

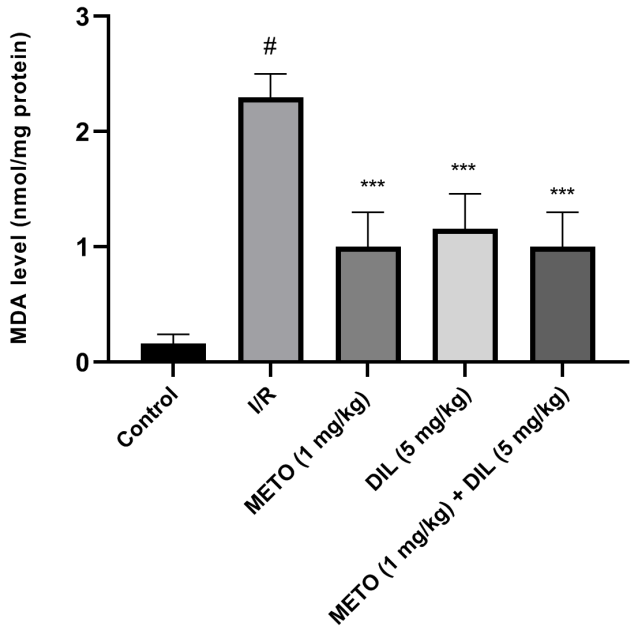

FIGURE 4 - The effects of pre-treatment with metoprolol (1 mg/kg/day) and diltiazem (5 mg/kg/day) alone and in combination on MDA level in the brain tissue; data are expressed as mean±S.E.M. N=6. # P<0.001 vs. control group. *** P<0.001 vs. I/R group using one-way ANOVA with *Tukey* post-test. I/R: Ischemia/reperfusion; METO: Metoprolol; DIL: Diltiazem.

DISCUSSION

This study demonstrates that pre-treatment with metoprolol and diltiazem effectively protects the brain tissue from ischemia and reperfusion-induced injuries. These positive effects appear as a reduction in cerebral infarct size, leukocyte accumulation, MPO activity, and MDA level in the brain tissues. The protection can be partially attributed to the prevention of oxidative stress and anti-inflammatory responses.

Studies have shown that cerebral I/R contributes to nerve damage and oxidative stress through calcium ion accumulation inside the cells. Intracellular calcium overload initiates a series of cytoplasmic and nuclear events. These events, consequently, result in activation of proteases and endonucleases, mitochondrial damage, acidosis, and free radical production, leading to nerve damage and apoptosis or necrosis, and consequently neural dysfunction (Kalogeris *et al*., 2012). These findings suggest that diltiazem, a voltage-dependent calcium channel blocker, might provide neuroprotection during I/R. In addition, inflammation is one of the most essential pathological factors in cerebral ischemia, characterized by neutrophil infiltration. Following an ischemic injury, activated leukocytes and endothelial cells release free radicals and inflammatory cytokines (Nour, Scalzo, Liebeskind, 2013). Finally, since cerebral ischemia in rats significantly increases the size of cerebral infarction, this size is a factor for measuring tissue's necrotic area (Zhang *et al*., 2014). A study demonstrated that diltiazem possesses antioxidant properties, prevents oxidative stress induced by dementia, and has potent neuroprotective effects. In general, the protective effects of diltiazem can be primarily attributed to the prevention of cytosolic calcium overload (Rani *et al*., 2015).

Moreover, previous studies have reported the antioxidant property of diltiazem, including protection against lipid peroxidation and the inhibition of the toxic effects of oxygen-derived free radicals. It has been shown that metoprolol has protective effects in acute cerebral ischemia, through maintaining redox homeostasis of aminothiols in plasma and brain and reducing oxidative stress (Ivanov *et al*., 2018). In other studies, the researchers found that metoprolol prevents cardiac oxidative stress associated with myocardial I/R injury and decreases infarct size (Kalaycioglu *et al*., 1999; Bao *et al*., 2015). The neuroprotective effects of metoprolol may be due to interference with calcium entry by decreasing the number of receptors (Iwata *et al*., 2010). These observations are in line with the results of our study. In the present study, also, MDA levels decreased as an indicator for lipid peroxidation and oxidative stress. Diltiazem inhibits interleukin 6 (IL-6) release and stimulates the production of anti-inflammatory mediator IL-10 at the end of cardiopulmonary bypass in patients undergoing coronary artery bypass (Fansa *et al*., 2003). Elsewhere, neuroprotective and anti-inflammatory effects of diltiazem on spinal cord I/R have been reported (Fansa *et al*., 2009). Additionally, β_1-adrenoceptor inhibitors suppress catecholamine and adrenaline in brain injury, leading to anti-inflammatory properties (Ley *et al*., 2009; McNamee *et al*., 2010). Anti-inflammatory effect is another neuroprotective mechanism against I/R. Our histological results also showed an inflammation reduction in the pre-treatment

groups. Intercellular adhesion molecules (ICAM) are important ligands for the adhesion of neutrophil and endothelial cells. ICAM1 expression increases during reperfusion and enhances neutrophil and endothelial cell infiltration (Ashabi *et al*., 2017). It has been concluded that adrenaline increases ICAM expression level in monocytes *in vitro,* and this effect is partly mediated by the β-adrenergic receptor. Furthermore, β-adrenoceptor antagonists reduced the infiltration and degranulation of neutrophils in the myocardium in experimental studies (Gao *et al*., 2000). The results of another study showed that metoprolol decreased MPO activity, indicating a reduction in neutrophil infiltration in damaged tissue after spinal cord injury in rats (Beril Gok *et al*., 2007). Metoprolol also inhibits the production of free oxygen radicals, thereby blocking the production of 4-hydroxynonenal (4-HNE) and reducing neutrophil chemotaxis. Metoprolol may decrease neutrophil migration through its inhibitory effect on protein kinase C (PKC) activity (Dunzendorfer, Wiedermann, 2000; Clemente-Moragón *et al*., 2020), which is consistent with our results. It has been demonstrated that diltiazem increases regional cerebral blood flow (rCBF) in marginal areas of ischemia and reduces the amount of H2O in the brain after an acute stroke. In vitro studies showed that cerebral vessels are highly sensitive to calcium channel blockers (Roy *et al*., 1985). Another study concluded that cortical infarct volume was lower in rats receiving β-adrenoceptor antagonists and improved neurological outcomes (Goyagi, Nishikawa, Tobe, 2010). In the present study, also TTC staining results proved a significant reduction in infarct size in the pre-treated groups. This reduction is another proof for neuroprotective effects of metoprolol and diltiazem in cerebral I/R injuries.

CONCLUSION

Our results showed that metoprolol and diltiazem can be considered neuroprotective agents in the global cerebral I/R injury and might protect patients who already use these medicines for any reason from the neurocerebral injuries caused by events such as stroke. Generally, these protective effects can be partially attributed to their anti-oxidative and anti-inflammatory properties.

CONFLICT OF INTEREST

None declared

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None.

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