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Effects of Benidipine Hydrochloride on Ischemia Reperfusion Injury of Rat Brain

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ABSTRACT

AIM: To evaluate the neuroprotective effects of benidipine hydrochloride on the cerebral cortex tissues in rats exposed to cerebral ischemia-reperfusion (I/R) injury.

MATERIAL and METHODS: Twenty-four male Wistar albino rats were randomly divided into three groups, and administered benidipine hydrochloride (10 µg/kg/day) orally through a catheter for 2 h to form the study group (BIR group, n=8). The I/R procedure was performed in the rats of the IR group (n=8), and a sham group was formed to determine the normal structure of the cerebral cortex (n=8). Transient ischemia was performed by clamping the left common carotid artery for 2 h. Subsequently, reperfusion was applied for 12 h. Cerebral infarct volumes were measured and cerebral cortex tissue samples were analyzed histopathologically and biochemically by measuring malondialdehyde (MDA), total glutathione, cyclooxygenase 1 (COX-1), COX-2 and superoxide dismutase (SOD)

RESULTS: The infarct area was markedly reduced in the BIR group vs. the IR group. Histopathologically, neuroprotective effects of benidipine hydrochloride were observed in the cerebral cortex tissues. The mean malondialdehyde and COX-2 levels were statistically higher in the IR group; however, in the BIR group, these levels were within the normal limits. Furthermore, the mean total glutathione, COX-1 and SOD levels were markedly lower in the IR group, and within the normal limits in the BIR group.

CONCLUSION: Benidipine hydrochloride may play a certain protective role in cerebral I/R injury. This effect may be related to improvement in the antioxidant capacity of brain tissue, and the inhibition of overproduction of inflammatory cytokines.

KEYWORDS: Cerebral ischemia reperfusion, Neuroprotection, Benidipine hydrochloride, Malondialdehyde, Glutathione, Rat

ABBREVIATIONS: I/R: Ischemia/reperfusion, **MDA:** Malondialdehyde, **tGSH:** Total glutathione, **COX:** Cyclooxygenase, **SOD:** Superoxide dismutase

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INTRODUCTION

Cerebral ischemia is one of the leading causes of death worldwide, following cardiovascular diseases and cancer. It accounts for approximately 6.3 million deaths annually (18,26). Generally, an ischemic stroke occurs as a result of atherosclerosis and, less frequently, brain trauma, which may lead to disruption of the structural integrity of cerebral blood vessels (14). Approximately two million brain cells die every minute after occlusion of a cerebral artery (29). Previous studies have reported that approximately 25% of the patients may have neurologic worsening during the first 24–48 h after stroke, and early mortality rate is approximately 15% (17,27). One of the principles of treatment in the clinic for cerebral ischemic injury is restoration of the blood reperfusion as early as possible. Some studies have mentioned that reperfusion should be achieved within 4.5 hours (17,21). However, reperfusion itself maycause brain injury, cerebral edema, brain hemorrhage, and neuronal death (13). This phenomenon is termed ischemia/reperfusion (I/R) injury (11,30). Numerous mechanisms have been linked to the pathological process of I/R injury, such as tissue damage caused by free radicals due to oxidative stress, energy metabolism disorder of brain tissues, toxicity of excitatory amino acids, increase in intracellular calcium, cytotoxic effects of nitric oxide, and damage in the blood–brain (BB) barrier (29,34,36). Oxidative stress is closely related to the damage caused by the excessive production of inflammatory cytokines. It results in the increased production of reactive oxygen species (ROS) and pro-inflammatory and inflammatory cytokines (8). Previous studies have demonstrated the protective effect of benidipine hydrochloride, a third generation and long-lasting dihydropyridine-derived calcium channel blocker, against cell injury during hypoxia and I/R (20,24,30). Treatment with benidipine hydrochloride inhibited the increase in lipid peroxidation and showed antioxidant activity and an anti-apoptotic nature (20,29). Thus, in the present study, we hypothesized that tissue damage due to cerebral I/R injury may be controlled bybenidipine hydrochloride. Therefore, in this study, cerebral infarct volumes of rats exposed to I/R injury were measured and histopathological examinations were performed. Intercalarily, the levels of biochemical parameters indicating the presence of oxidative stress, inflammation, and antioxidant markers, such as malondialdehyde (MDA), total glutathione (tGSH), cyclooxygenases 1,2 (COX-1, COX-2) and superoxide dismutase enzyme (SOD) were measured.

MATERIAL and METHODS

This study was performed at the Erzincan University School of Medicine Experimental Research Center, Erzincan, Turkey. All surgical protocols were approved by the Institutional Animal Care and Use Committee of Atatürk University, Erzurum, Turkey (No: 11/197-25.10.2018).

Animals

The animals were maintained in polypropylene cages under a 12/12-hour light/dark cycle and controlled temperature (18°C–22°C) prior to and during the experiment. All rats were fed with pellet rat food (Bil-Yem Ltd., Ankara, Turkey) and water.

A total of 24 adult male Wistar albino rats weighing 290–305 g were randomly divided into the following three groups:

Sham group (SG): Sham operation was performed to determine the normal structure of the cerebral cortex (n=8).

IR group: The I/R procedure was performed on the animals of this group (n=8).

BIR group: Benidipine hydrochloride (10 µg/kg/day, for 2 h) was administered orally to the animals of this group before I/R procedure (n=8).

Experimental Design

Body temperatures of animals were continuously monitored using a rectal probe, and were regulated at 37 ± 0.5°C using a homoisothermy bench. Benidipine hydrochloride (10 µg/kg/day; Deva, Turkey) was administered orally through a catheter for 2 hours. An equal volume of distilled water was administered orally to the animals of the IR group and SG. Subsequently, all animals were anesthesized with 25 mg/kg of thiopental sodium (IE Ulagay, Turkey). After superficial microdissection, a deep microdissection was performed toward the left carotid artery. The trachea was observed and the paratracheal muscles were dissected. The left common carotid artery was cross-clamped with an aneurysm clip (Yasargil FE 721; Aesculap, USA). Regional cerebral blood flow was measured with laser-Doppler flowmetry (PF5010; Perimed Co.Ltd., Sweden). A reduction (≥90%) in regional cerebral blood flow indicated successful occlusion.The layers were closed with a sterile surgical thread, and all animals were placed into individual cages. Following a 2-hour rest, all animals were re-anesthetized with 25 mg/kg of thiopental sodium and clamps were removed. After 12 hours of reperfusion, the animals were sacrificed using high-dose anesthesia, and the brains were carefully removed from the skull. Animals in the SG underwent the same operative procedure with the exception of artery occlusion.

Evaluation of the Infarct Area

Coronal slices (thickness: 2 mm) were obtained and stained at 37°C for 30 minutes in 2% triphenyltetrazolium chloride (Sigma–Aldrich; Merck KGaA, Darmstadt, Germany) to calculate the brain infarct volumes. Infarct volume was detected using image analysis software (Image Pro plus 6.0 system; Media Cybernetics, Inc., Rockville, MD, USA). Infarct and noninfarct areas were traced using the photographs of triphenyltetrazolium chloride-stained brain slices (ImageJ 1.48; National Institutes of Health, Bethesda, MD, USA). Firstly, the swelling area was calculated according to the following formula: ipsilateral hemisphere area–contralateral hemisphere area. The infarct area was evaluated with the following formula:

infarct area (%) = (contralateral area– [(ipsilateral area – swelling area) – ipsilateral noninfarct area)] /contralateral area.

Histopathological Examination

For the histopathological examination of the cerebral cortex,

tissue samples were identified in a 10% formaldehyde solution for assessment using light microscopy. Following the identification process, tissue samples were washed under tap water in cassettes for 24 hours. Samples were subsequently treated with alcohol (70%, 80%, 90%, and 100%) to dehydratethe tissues. Tissues were passed through xylol and embedded in paraffin. Sections (4–5 µm) were cut from the paraffin blocks and hematoxylin–eosin (H&E) staining was performed. Images were captured following assessment with the Olympus DP2-SAL firmware program (Olympus® Inc., Tokyo, Japan). Histopathological assessment was performed by a pathologist blinded to the study groups.

Biochemical Examination

For biochemical examination, saline was added to the tissues at a ratio of 1:9 in an ice-water bath at 4°C, homogenized with a high-speed homogenizer, and centrifuged at 4,000 r/min for 10 minutes. Finally, the supernatant was removed, divided into aliquots, and stored at –20°C in a refrigerator for further use.

MDA Analysis

MDA measurements were based on the method described by Ohkawa et al. (23), involving spectrophotometrical measurement of absorbance of the pink-colored complex formed by thiobarbituric acidand MDA. The serum/tissue-homogenate sample (0.1 mL) was added to a solution containing 0.2 mL sodium dodecyl sulfate (80 g/L), 1.5 mL acetic acid (200 g/L), 1.5 mL 2-thiobarbiturate (8 g/L), and 0.3 mL distilled water. The mixture was incubated at 95°C for 1 h. Upon cooling, 5mL n-butanol:pyridine (15:1) was added. The mixture was vortexed for 1 min and centrifuged for 30 minutes at 4,000 rpm. The absorbance of the supernatant was measured at 532 nm. The standard curve was obtained using 1,1,3,3-tetramethoxypropane.

tGSH Analysis

tGSH analysis was performed according to the method defined by Sedlak and Lindsay (29). Reduction of the sulfhydryl groups of 5,5'-dithiobis [2-nitrobenzoic acid] (DTNB) disulfide, a colorful compound, leads to the production of a yellow-colored compound. The yellow color produced during the reduction was measured through spectrophotometry at 412 nm. For this measurement, a cocktail solution (5.85 mL sodium-phosphate buffer [100 mM], 2.8 mL DTNB [1 mM], 3.75 mL nicotinamide adenine dinucleotide phosphate [1 mM], and 80 µL GSH reductase [625 U/L]) was prepared. Prior to measurement, 0.1 mL meta-phosphoric acid was added to 0.1 mL serum/tissue-homogenate, and the mixture was centrifuged for 2 min at 2,000 rpm for deproteinization. The 0.15 mL cocktail solution was added to 50 µL of supernatant. Measurements were performed with GSH disulfide according to the standard graphic at 412 nm.

Measurement of COX Activity: Preparation of Reagents for COX Activity Analysis

COX activity analysis was performed according to the method defined by Kulmacz and Lands (17). The analysis buffer was prepared by diluting 3 mL of the stock analysis buffer in 27 mL of high-performance liquid chromatography-grade water, which is free from organic/inorganic compounds and does not exhibit ultraviolet absorbance. The hem reagent was prepared by diluting 88 µL hem solution in 1.912 mL of the previously prepared analysis buffer. The arachidonic acid solution was prepared by adding 100-µL potassium hydroxide to 100-µL arachidonic acid, vortexing the resultant solution, and diluting it with 1.8 mL high-performance liquid chromatography-grade water. Other reagents used were the COX standard, colorimetric substrate, DuP697 (COX-2 inhibitor), and SC-560 (COX-1 inhibitor), which are commercially available. The activity of COX in the tissue was expressed as nmol/min/mg protein (U/mg protein).

SOD Analysis

SOD analysis was performed according to the method of Sun et al (31). When xanthine is converted to uric acid by xanthine oxidase, SOD is formed. If nitro blue tetrazolium is added to this reaction, SOD reacts with nitro blue tetrazolium and a purple formazan dye is formed. The sample was weighed and homogenized in 2 ml of 20 mmol/L phosphate buffer containing 10 mmol/L ethylene diamine tetraacetic-acid (EDTA) at pH 7.8. The sample was centrifuged at 6000 rpm for 10 minutes and than the brilliant supernatant was used as assay sample. The measurement mixture containing 2450 µL measurement mixture (0.3 mmol/L xanthine, 0.6 mmol/L EDTA, 150 µmol/L NBT, 0.4 mol/L Na_2CO_3, 1 g/l bovine serum albumin), 500 µL supernatant and 50 µL xanthine oxidase (167 U/l) was vortexed. Then it was incubated for 10 minutes. At the end of the reaction, formazan occured. The absorbance of the purple-colored formazan was measured at 560 nm.

Statistical Analysis

Continuous variables are presented as mean ± standard deviation. The normality of data distribution was confirmed using the Shapiro–Wilk test. For comparison of the threegroups, oneway analysis of variance was used. As a *post-hoc* test, Tukey's honestly significant difference or the Games–Howell test was used according to the homogeneity of variances. In graphical representations, the mean values and standard errors are shown. The statistical level of significance for all tests was considered to be 0.05. Statistical analysis was performed using the IBM SPSS Version 20.0 software (IBM Corp., Armonk, NY, USA).

RESULTS

Benidipine Hydrochloride Reduced the Infarct Area

The border between normal and infarcted tissue was well-defined, and could be easily detected by gross inspection (Figure 1A, B). Marked reduction in the infarct area was observed in the BIR group vs. the IR group. In the IR group, the mean infarct area was 59.5 ± 7.1 mm^2 (35.28% ± 4.13%). In the BIR group, the mean infarct area was markedly reduced to 39.1 ± 4.4 mm^2 (23.56% ± 3.47%) ($p<0.05$). In the SG, there was no infarct site detected.

Histopathological Findings

According to the microscopic evaluations of cerebral cortex

tissues, in the SG, the structure and morphology of neurons, glial cells, and blood vessels was normal (Figure 2). However, in the IR group, pericellular edema in neurons, abnormal morphological changes and degenerations in astrocytes, increased microglial cell presence which suggested apoptosis in the cortex neuronal/glial cells, apoptotic bodies and dilated and congested blood vessels were observed (Figure 3). In the BIR group, amelioration of the cerebral cortex attracted our attention. Normal intensity of microglial cells, decreased congestion in blood vessels, absence of pericellular edema, and no degeneration in astrocytes were observed (Figure 4).

Biochemical Findings

The mean MDA levels were significantly higher in the IR group vs. the SG and BIR group ($p<0.001$ and $p<0.001$, respectively); there was no significant difference between the SG and BIR group ($p=0.157$) (Figure 5).

The mean COX-2 levels were significantly higher in the IR group vs. the SG and BIR group ($p<0.001$ and $p<0.001$, respectively); there was no significant difference between the SG and BIR group ($p=0.090$) (Figure 6).

The mean tGSH levels were significantly higher in the IR group vs. the SG and BIR group ($p<0.001$ and $p<0.001$, respectively); there was no significant difference between the SG and BIR group ($p=0.106$) (Figure 7).

The mean COX-1 levels were significantly lower in the IR group vs. the SG and BIR group ($p<0.001$ and $p<0.001$, respectively);

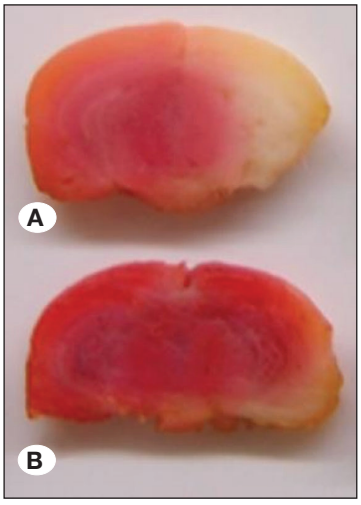

Figure 1: Triphenyltetrazolium chloride staining of coronal brain sections. Infarct areas are indicated by lines. **A)** Image of a coronal slice of the brain obtained from the IR group. **B)** Image of a coronal slice of the brain obtained from the BIR group.

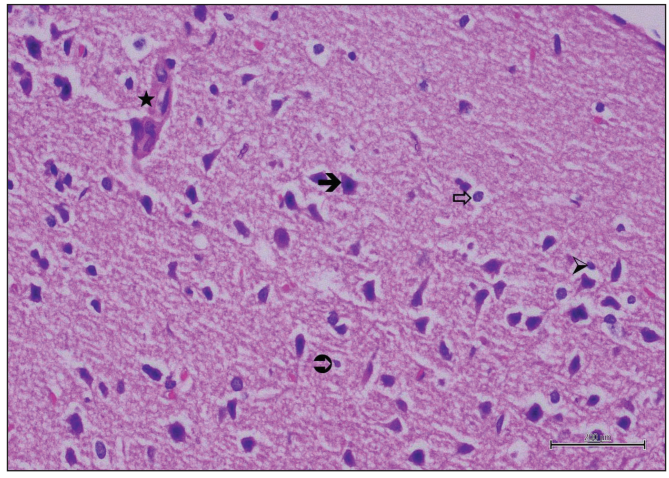

Figure 2: Hematoxylin–eosin staining of cerebral cortex tissue obtained from the SG. The structure and morphology of neurons, glial cells, and blood vessels were normal. ➔**:** neuron; ★**:** blood vessel; ⇨**:** astrocyte; ➢**:** oligodendrocyte; ⊃**:** microglia; ×200.

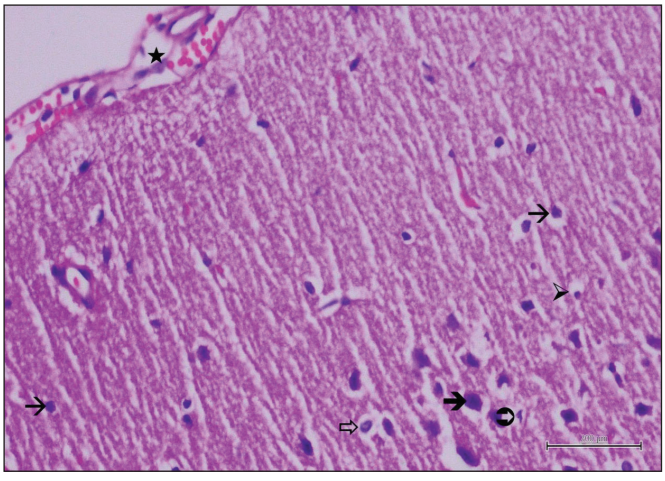

Figure 3: H&E staining of cerebral cortex tissue obtained from the IR group. Pericellular edema in neurons, morphological changes and degenerations in astrocytes, apoptosis in the neurons / glial cells and increased presence of microglial cells were observed. These findings suggested apoptosis in the neuronal/glial cells of the cortex. Moreover, dilated and congested blood vessels were observed. ➔**:** degenerated neuron; ⇨**:** degenerated astrocyte; ➢**:** oligodendrocyte; ⊃**:** increasing number of microglia; ➔**:** apoptotic bodies ★**:** congested blood vessel; ×200.

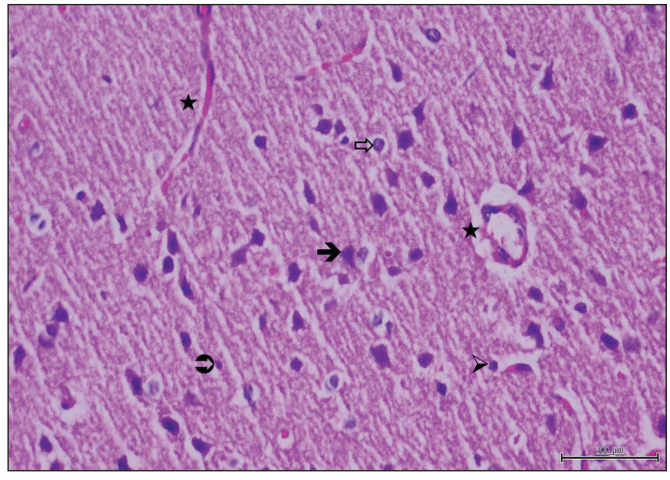

Figure 4: H&E staining of cerebral cortex tissue obtained from the BIR group. Normal presence of microglial cells, decreased congestion in blood vessels, and absence of pericellular edema were observed. ➔**:** neuron; ⇨**:** normal shape of astrocyte; ➢**:** normal shape of oligodendrocyte; ⊃**:** decreased number of microglial cells; ★**:** normal blood vessel; ×200.

there was no significant difference between the SG and BIR group (p=0.203) (Figure 8).

The mean SOD levels was found statistically lower in the IR group than SG and BIR group (p<0.001 and p<0.003, respectively) and there was no significiantly difference between SG and BIR groups (p=0.788) (Figure 9).

DISCUSSION

In the present study, we investigated the neuroprotective effects of benidipine hydrochloride on cerebral I/R injury in rats. Benidipine hydrochloride (10 µg/kg/day) was administered orally to the BIR group for 2 hours. This dose was selected based on previous studies, which demonstrated that administration of benidipine hydrochloride at this dose markedly reduced calcium overload and decreased post ischemic apoptosis (8,20)**.** The cerebral I/R injury model was produced by cross-clamping the carotid artery using an aneurysm clip. Alternative methods, such as intravascular thread occlusion and balloon injury, are also available.

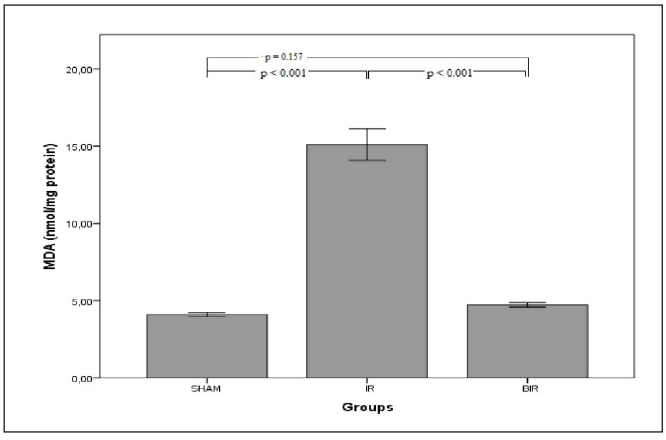

Figure 5: Comparison of malondialdehyde (MDA) levels between groups.

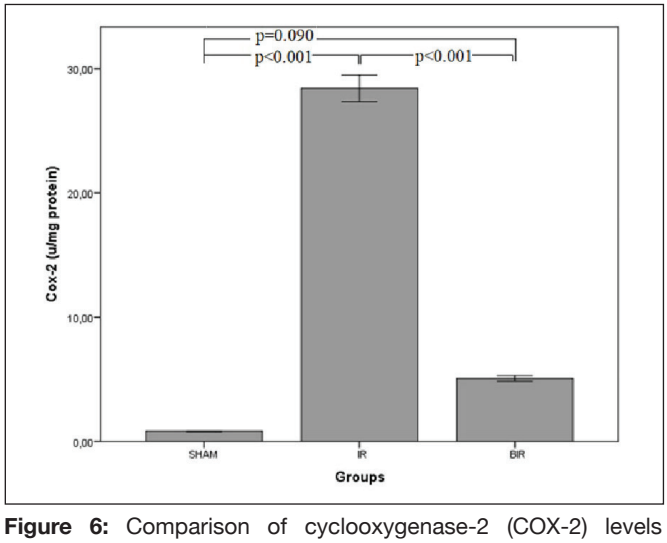

Figure 6: Comparison of cyclooxygenase-2 (COX-2) levels between groups.

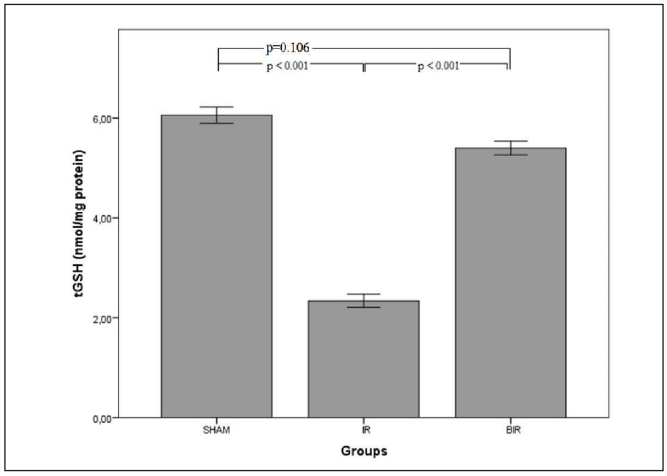

Figure 7: Comparison of total glutathione (tGSH) levels between groups.

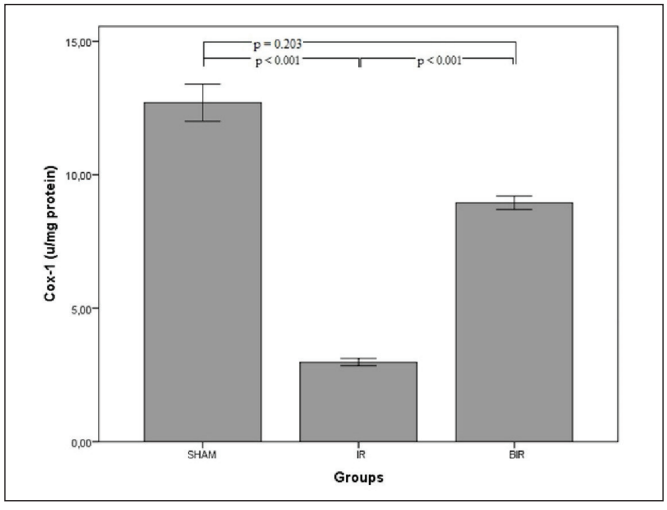

Figure 8: Comparison of cyclooxygenase-1 (COX-1) levels between groups.

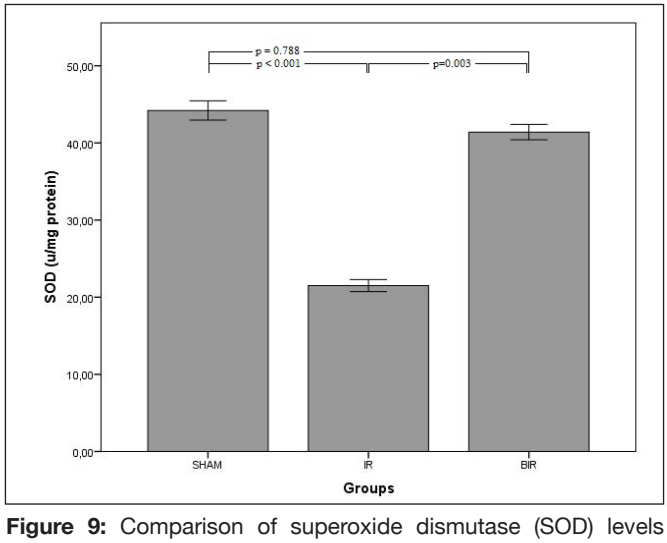

Figure 9: Comparison of superoxide dismutase (SOD) levels between groups.

The vascular clamping model has been safely used in numerous studies and is reported to provide >90% vascular occlusion (27)**.** After I/R injury, the infarct areas were measured and compared between the groups. According to previous studies, measurement of the infarct area has been one of the specific markers for evaluating brain injury (18,19,26). We calculated the infarct areas by considering the peri-infarct edema areas to investigate the true infarct area, based on the method describedby McBride et al. (21). According to our findings, benidipine hydrochloride markedly reduces the brain infarct area. To the best of our knowledge, there are no studies in the literature investigating the protective effect of benidipine hydrochloride on cerebral infarction. According to a study conducted by Ikeda et al. in rats (10), the mean arterial blood pressure and cerebral vascular resistance were lowered after treatment with benidipine hydrochloride. Therefore, in that study, the investigators reported that benidipine hydrochloride is effective in cerebrovascular disorders by its regulation of cerebral blood flow. Moreover, Shirakura et al. (30) in their study reported that neurological symptoms (e.g., ataxia, convulsions, and loss of righting reflex) were improved, and survival time was prolonged by pretreatment with benidipine hydrochloride after occlusion in rats of the bilateral common carotid arteries. The role of apoptosis in I/R injury has been established, and considerable evidence has indicated that calcium plays a critical role in the signal transduction pathway, leading to apoptosis. It has previously been shown that benidipine hydrochloride attenuated post ischemia apoptosis by inhibiting mitochondrial cytochrome c release, caspase-9 activation, and subsequent caspase-3 activation (11,20). Based on the histological results, there was no pericellular edema in neurons, degeneration in astrocytes, or dilated and congested vascular structures observed in the rats pretreated with benidipine hydrochloride. The relationship between cell damage and increased intracellular calcium was established in previous studies (7,11,16,22). Several mechanisms have been reported that are related to the protective effect of benidipine hydrochloride on cells. Different studies have consistently demonstrated that calcium overload after I/R increases damage in brain microcirculation (18,26). According to our biochemical results, treatment with benidipine hydrochloride prevented the increase in the levels of MDA in cerebral cortex tissues that were exposed to I/R. MDA is a 3-carbon, low-molecular-weight aldehyde produced by a free radical-mediated chain of reactions. It is largely used as a marker of lipid peroxidation and oxidative stress (2,9). As known, the I/R event causes increased intracellular calcium (Ca^{+2}) in the tissues (2). And increased intracellular Ca^{+2} activates Ca^{+2} dependent cytosolic proteases (5). Cytosolic proteases convert intracellular hypoxanthine dehydrogenase to xantine oxidase which is the main source of ROS. As a result, ROS production increaes and oxides the cell membrane lipids, resulting in toxic products such as MDA (15,28). This information shows that I/R causes damage by increasing ROS production in the tissues. Benidipine hydrochloride is a L-type channel Ca+2 blocker, suggesting that it may have prevented increased ROS production in the brain tissues and protected them from oxidative damage. Furthermore, under normal conditions, COX-2 is not expressed in most cells. In the present study, higher levels of COX-2 were found in the cerebral cortex tissues of rats in response to I/R injury. Nevertheless, following pretreatment with benidipine hydrochloride, the levels of COX-2 were within normal limits. It has been known that elevated levels of COX-2 are related with oxidative stress and inflammation in the tissues (17). One of the important mechanisms of I/R damage is activation of phospholipase A2 by intracellular Ca^{+2} increase during ischemia (6,35). Phospholipase A2 increases arachidonic acid production from membrane phospholipids. Then COX-2 activates and releases pro-inflammatory prostoglandins and ROS species from arachidonic acid (6,35). The fact that benidipine hydrochloride inhibited I/R related COX-2 activity increase may be due to the fact that it blocked the entry of Ca^{+2} ions into the cell during ischemia. And as known over-produced ROS are neutralized by GSH and other endogenous antioxidants (32). Particularly, the defensive system of the human body which includes such antioxidant enzymes as SOD and GSH, has a key role in the resistance of the neuronal cells to ROS-induced cell death (9). If antioxidants fail to neutralize ROS, their amount decreases and oxidative tissue damage occurs (15). GSH acts as a cofactor in enzyme reactions and provides storage for cysteine and regulates cellular events including gene expression, DNA and protein synthesis, cytokine production, immune response and protein glutathionylation (25). Therefore there is a considerable interest in this molecule and in the measurement of it in vivo. Benidipine hydrochloride may reduce the consumption of GSH by inhibiting the increase of ROS by Ca^{+2} channel blockade in tissues exposed to I/R. In addition, SOD, which is one of the markers of defense against tissue damage caused by ROS, catalyzes the dismutation of superoxide anion to hydrogen peroxide and prevents the formation of the hydroxyl radicals (12). So SODs have been reported to alleviate inflammatory, respiratory, metabolic, cardiovascular diseases and central nervous system disorders in the literature (3). As a sample, Ohno et al. mentioned the cardiovascular protective effect of benidipine in their study and thay stated that this agent increased SOD levels in the tissues (24). And this mechanism had been reported to be achieved by Ca^{+2} channel blockade in addition to its antihypertensive efficacy. Also traditionally, COX-1 has been considered as the constitutively expressed isoform, primarily responsible for the baseline prostaglandin production (14). According to the previous studies COX-1 is thought to produce prostaglandines important for homeostasis and certain physiological functions (4,33). Recent data suggest that COX-1 facilitates activation of glial cells and supports inflammatory processes (14). Therefore, it is important that benidipine maintains COX-1 levels within normal limits.

Limitations

The neurological status and movement analysis of the rats has not been analyzed in this study. This is the most important limitation of this study. In addition, brain infarction rates were compared with the control group, effective results were found, but blood pressure measurements could not be performed for antihypertensive efficacy which is thought to be one of the mechanism of action.

CONCLUSION

The protective effects of benidipine hydrochloride on cells have been shown in numerous studies. Although a mechanism of protection for endothelial cells has been mentioned, the protective effects on neural cells have not yet been investigated. The findings of the present study provide a new direction in this field by presenting the potential mechanisms of this neuroprotective action.

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