Valsartan modulates the inflammatory response and apoptosis and protects from cerebral ischemia Reperfusion injury

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ABSTRACT

Objectives: The objective of this study is to investigate the possible cerebroprotective potential of valsartan in brain ischemia reperfusion injury via interfering with inflammation and oxidative pathway and apoptosis.

Materials and Methods: Adult albino rats were randomized into four groups as follow: <u>Group (1)</u> sham group: the rats were subjected to the same surgical procedures as other groups but the common carotid arteries were not occluded; <u>Group (2)</u> control (ischemic-reperfused) group: the rats were subjected to the same surgical procedures as other groups with bilateral common carotid artery occlusion (BCCAO) for 30 min. followed by reperfusion for 1 hr but without drug; <u>Group (3)</u> control vehicle group: ten days before surgery, rats received daily the vehicle of valsartan drug, normal saline (0.9% Nacl) (1 ml/kg/day) intraperitoneally, then anesthesia and surgery with BCCAO for 30 min. followed by reperfusion for 1 hr were done and <u>Group (4)</u> valsartan treated group: rats received daily valsartan intraperitoneally. The dose of valsartan was (3 mg/kg /day) for ten days before the surgery, then anesthesia and surgery with BCCAO for 30 min. followed by reperfusion for 1 hr were done and <u>Group (4)</u> or alsartan treated group: rats received daily valsartan intraperitoneally. The dose of valsartan was (3 mg/kg /day) for ten days before the surgery, then anesthesia and surgery with BCCAO for 30 min.

Results: At the end of the experiment, the levels of cerebral TNF- α , IL-6, IL-10,Caspase-3,Bax,MDA,CD4 and MPO significantly (p < 0.05) increased in control group as compared with the sham group and the level of cerebral GSH significantly (p < 0.05) decreased in control group as compared with the sham group, while there was insignificant difference in cerebral levels of CD8 between the four experimental groups. Histopothological analysis showed that rats in control group showed significant cerebral injury. Treatment with valsartan significantly counteracted the increase in the cerebral levels of TNF- α , IL-6, IL-10,Caspase-3,Bax,MDA,CD4 and MPO and the decrease of GSH .Histopathological analysis revealed that valsartan significantly (P < 0.05) reduced the severity of cerebral injury in the rats underwent BCCAO.

Conclusions: The results of the study revealed that inflammatory cytokines, apoptosis pathways and oxidative stress mediators are involved in global cerebral ischemia induced by bilateral common carotid artery occlusion. Cerebral ischemia reperfusion injury can be modified by valsartan via its anti-inflammatory, anti-apoptosis and anti-oxidant effects.

Keywords: Cerebral ischemia reperfusion injury, inflammation, apoptosis, oxidative stress, valsartan

Introduction

The term ischemia reperfusion injury (IRI) describes the experimentally and clinically prevalent finding of tissue ischemia with inadequate oxygen supply followed by successful reperfusion initiates a wide and complex array of inflammatory responses that may both aggravate local injury as well as induce impairment of remote organ function ¹. Ischemiareperfusion injury contributes to the pathophysiology of many conditions, include the different forms of acute vascular occlusions such as stroke, myocardial infarction, peripheral vascular insufficiency and hypovolemic shock With the relevant reperfusion strategies like thrombolytic therapy, coronary

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angioplasty, cardiopulmonary bypass and operative revascularization ². Cerebral ischemia leads to energy depletion and cell death, which can stimulate immune responses, leading to inflammatory cells activation and infiltration. Reperfusion of the occluded vessel, either spontaneously or by the collateral circulation or by therapeutic recanalization, leads to the generation of reactive oxygen species (ROS) that are delivered with the reperfused oxygenated blood or produced within brain and immune cells. ROS can then stimulate ischemic cells, even ischemic neurons, to secrete inflammatory chemokines and cytokines that enhance the biosynthesis of adhesion molecule in the cerebral vasculature and also lead to peripheral leukocyte recruitment ^{3.} As inflammatory cells become activated, they can release a variety of cytotoxic molecules including more cytokines, matrix metalloproteinases (MMPs), nitric oxide (NO) and more ROS. These molecules may provoke more cell damage as well as disruption of the extracellular matrix and blood-brain barrier (BBB) ^{4,5}. Secondary ischemic brain damage occurs as a consequence of brain edema and postischemic microvascular stasis leading to hypoperfusion and post-ischemic inflammation ^{6,7}. The recruitment of peripheral circulating leukocytes into the brain parenchyma can produce an augmentation of inflammatory signal cascades, which will enhance the damage. These processes are mainly prominent during reperfusion which is often associated with microvascular injury, particularly due to increased permeability of capillaries and arterioles that lead to an increase of fluid filtration across the tissues. These activated endothelial cells produce more ROS following reperfusion, and results in a subsequent inflammatory response. White blood cells, carried to the area by the newly returning blood, release a mass of inflammatory factors such as interleukins (ILs) as well as free radicals in response tissue damage. The restored blood flow to reintroduces oxygen within cells that damages cellular proteins, DNA, and the plasma membrane. Damage to the cell's membrane may in turn causes the release of more free radicals. ROS may also act indirectly in redox signaling to turn on apoptosis. White blood cells may also bind to the endothelium of small capillaries, obstructing them and leading to more ischemia ^{8,9}. Early restoration of blood flow remains the treatment of choice for limiting brain injury following ischemic stroke. Improved educational efforts that emphasize the early signs and symptoms of stroke, coupled with the widespread application of thrombolytic therapy to patients with acute ischemic stroke have increased the number of patients benefiting from reperfusion ¹⁰. While reperfusion of the ischemic brain is desirable, tissue damage may result from reperfusion only. Reperfusion appears to enhance the inflammatory response and causes additional injury to adjacent brain tissue ¹¹. From experimental stroke, blocking various aspects of the inflammatory cascade has shown to improve injury ⁸. valsartan is an angiotensin II receptor antagonist (ARB) with particularly high affinity for the angiotensin type I (AT_1) receptor. valsartan has shown helpful effects on vasoconstriction, proliferation, endothelial function, remodeling and thrombogenesis, inflammation and atherosclerosis ¹².

Materials and methods Animals

The study was performed using 24 Adult albino rats weighting (200-250 g), provided by the animal house of high institute of infertility diagnosis and assisted reproductive technologies / Al-nahrain University. The rats were housed in the animal house of College of pharmacy/ Kufa University, in a room in which lighting was controlled (12 hr on, 12 hr off), temperature was kept at (25±1°C) and humidity was kept at (60–65%) with unlimited access to food and water until the start of experiments. The Animal Investigation Committee(AIC) office of Kufa university approved the experimental protocol.

Preparation of valsartan

Valsartan was supplied by (Pioneer co. Sulaymanieyah/Kurdistan Iraq), and was prepared immediately before use by dissolving it in normal saline.

Experimental groups

After one week of acclimatization, the rats were divided randomly into four groups (6 rats in each group) as follow: Group(1) sham group: The rats were subjected to the same surgical procedures as other groups but the common carotid arteries were not occluded; Group(2)control (ischemic-reperfused) group: The rats were subjected to the same surgical procedures as other groups with bilateral common carotid artery occlusion (BCCAO) for 30 min. followed by reperfusion for 1 hr but without drug; Group(3)control vehicle group:

Ten days before surgery , rats received daily the vehicle of valsartan drug, normal saline (0.9% Nacl) (1 ml/kg/day), intraperitoneally (iP) ¹³ then anesthesia and surgery with BCCAO for 30 min. followed by reperfusion for 1 hr were done; Group(4) valsartan treated group: The rats received daily valsartan

intraperitoneally (iP). The dose of valsartan was (3 mg/kg /day) for te days before the surgery ¹⁴, then anesthesia and surgery with BCCAO for 30 min. followed by reperfusion for 1 hr were done.

Induction of global brain ischemia

Each rat was anesthetized by intraperitoneal (iP) injection of 100 mg/kg of ketamine and 5 mg/kg of xylazine ¹⁵. Within few min, the rat became unconscious, then placed in supine position and exposed to light source to keep it worm. After that a midline ventral small skin incision in the neck was made and the paratracheal muscles and fascia were splitted and pulled by stay sutures to expose the trachea, carotid arteries and vagal nerves. Both common carotid arteries were exposed, with special attention paid to separate and preserve the vagus nerve fibers and global cerebral ischemia was induced by BCCAO ¹⁶ by using vascular clamps for 30 min. After 30 min of global cerebral ischemia, the clamps were removed to allow the reflow of blood through carotid arteries (reperfusion) for 1 hr 17.

Preparation of samples

Tissue preparation for TNF- α , IL-6, IL-10, Caspase-3 ,Bax ,CD4,CD8, MPO-ANCA IgG, ,MDA and GSH measurement

After decapitation, the brain was removed and washed in cold normal saline (0.9% Nacl) to remove any blood or debris and subsequently blotted on filter paper. Afterward, brain tissues were homogenized in ice-cold 1:10 (w/v) 0.1 M phosphate-buffered saline (PBS) (pH 7.4), containing 1× protease inhibitor cocktail and 0.2% Triton X-100 for 30 seconds, using a high intensity ultrasonic liquid processor. The resulting homogenates were centrifuged at 15,000 *g* for 30 min, at 4°C, and supernatants were stored at -80° C until analysis was done ¹⁸.

Tissue preparation for histopathological analysis and Scoring of brain damage

After 30 min. ischemia and 60 min. reperfusion, decapitation was done and the brain was removed and fixed with 10% formalin and embedded in

paraffin wax and cut into coronal sections of 4-8µm thickness. The sections were stained with haematoxylin and eosin (H&E) dye for histopathological examination that was done by pathologist. The scoring system for the pathological changes in ischemia reperfusion injury is as follows 19 : 0:(normal) = no morphological signs of damage; 1:(slight) = edema or eosinophilic or dark neurons (pyknotic) or dark/ shrunk cerebellar Purkinje cells; 2:(moderate)= at least two small hemorrhages; 3:(severe) = clearly infarctive foci (local necrosis).

Statistical analysis

All data are expressed as mean \pm SEM. The difference between various groups were analyzed by one-way analysis of variance (ANOVA) followed by multiple comparison tests as Post Hoc. LSD. Non- parametric tests were used to assess the statistical significance of histopathological parameter. Cerebral lesions is a nonnormally distributed variable. The Fisher exact test is used when members of two independent groups can fall into one of two mutually exclusive categories. The test is used to determine whether the proportions of those falling into each category differ by group. In all tests *P*< 0.05 was considered to be statistically significant.

Results

Effect on inflammatory markers (TNF- α , IL-6, IL-10)

At the end of the experiment, the levels of cerebral TNF- α , IL-6, IL-10 significantly (P<0.05) increased in control group as compared with sham group. The levels of cerebral TNF- α and IL-6 of valsartan treated group were significantly (p<0.05) lower than that of control-vehicle group. The levels of cerebral IL-10 of valsartan treated group were significantly (p<0.05) higher than that of control-vehicle group. The values of cerebral TNF- α , IL-6, IL-10 are shown in figures (1, 2 and 3).



Figure (1): Error bar chart shows the difference in mean± SEM values of cerebral TNF- α level (pg/mg) in the four experimental groups at the end of the experiment (No. of animals = 6 in each group). * P < 0.05 vs. sham group, ** P < 0.05 vs. control-vehicle group.



Figure (2): Error bar chart shows the difference in mean± SEM values of cerebral IL-6 level (pg/mg) in the four experimental groups at the end of the experiment (No. of animals = 6 in each group). * P < 0.05 vs. sham group, ** P < 0.05 vs. control-vehicle group.



Figure (3): Error bar chart shows the difference in mean± SEM values of cerebral IL-10 level (pg/mg) in

the four experimental groups at the end of the experiment (No. of animals = 6 in each group). * P < 0.05 vs. sham group, ** P < 0.05 vs. control-vehicle group.

Effect on apoptptic markers (caspase-3 and Bax)

At the end of the experiment, the levels of cerebral caspase-3 and Bax significantly (P<0.05) increased in control group as compared with sham group. The levels of cerebral caspase-3 and Bax of valsartan treated group were significantly (p<0.05) lower than that of control-vehicle group. The values of cerebral caspase-3 and Bax are shown in figures (4 and 5).



Figure (4): Error bar chart shows the difference in mean± SEM values of cerebral caspase-3 level (pg/mg) in the four experimental groups at the end of the experiment (No. of animals = 6 in each group). * P < 0.05 vs. sham group, ** P < 0.05 vs. control-vehicle group.



Figure (5): Error bar chart shows the difference in mean± SEM values of cerebral Bax level (pg/mg) in the four experimental groups at the end of the experiment (No. of animals = 6 in each group). * P < 0.05 vs. sham group, ** P < 0.05 vs. control-vehicle group.

Effect on oxidative stress markers (MDA and GSH)

At the end of the experiment, the level of cerebral MDA significantly (P<0.05) increased in control group as compared with sham group, while the level of cerebral GSH significantly (P<0.05) decreased in control group as compared with sham group. The level of cerebral MDA of valsartan treated group was significantly (p<0.05) lower than that of control-vehicle group, while the level of cerebral GSH of valsartan treated group was significantly (p<0.05) higher than that of control-vehicle group. The values of cerebral MDA and GSH are shown in figures (6 and 7).



Figure (6): Error bar chart shows the difference in mean± SEM values of cerebral MDA level (pg/mg) in the four experimental groups at the end of the experiment (No. of animals = 6 in each group). * P < 0.05 vs. sham group, ** P < 0.05 vs. control-vehicle group.



Figure (7): Error bar chart shows the difference in mean± SEM values of cerebral GSH level (pg/mg) in the four experimental groups at the end of the experiment (No. of animals = 6 in each group). * P < 0.05 vs. sham group, ** P < 0.05 vs. control-vehicle group.

Effect on CD4+T-Lymphocytes

At the end of the experiment, the level of cerebral CD4+T-Lymphocytes significantly (P<0.05) increased in control group as compared with sham group. The level of cerebral CD4+T-Lymphocytes of valsartan treated group was significantly (p<0.05) lower than that of control-vehicle group. The value of cerebral CD4+T-Lymphocytes is shown in figure (8).



Figure (8): Error bar chart shows the difference in mean \pm SEM values of cerebral CD4+T-Lymphocytes level (pg/mg) in the four experimental groups at the end of the experiment (No. of animals = 6 in each group). * P < 0.05 vs. sham group, ** P < 0.05 vs. control-vehicle group.

Effect on CD8+T-Lymphocytes

At the end of the experiment, there was insignificant difference in cerebral level of CD8⁺T-Lymphocytes between the four experimental groups. The value of cerebral CD8⁺T-Lymphocytes is shown in figure (9).



Figure (9): Error bar chart shows the difference in mean± SEM values of cerebral CD8+T-Lymphocytes

level (pg/mg) in the four experimental groups at the end of the experiment (No. of animals = 6 in each group).

Effect on Myeloperoxidas-Antineutrophil Cytoplasmic Antibody IgG (MPO -ANCA IgG)

At the end of the experiment, the level of cerebral MPO significantly (P<0.05) increased in control group as compared with sham group. The level of cerebral MPO of valsartan treated group was significantly (p<0.05) lower than that of control-vehicle group. The changes in cerebral MPO is shown in table (1).

Table 1: Relation between valsartan and control vehicle groups regarding MPO.

	MPO +ve	MPO -ve	Total
Valsartan	1	5	6
Control vehicle	6	0	6
Total	7	5	12

The fisher exact statistic value is significant at p<0.05.

Histopathological findings

The cerebral injury was assessed in the rat's brain of the four experimental groups according to ¹⁹ and the results were as follow: In the sham group, a cross sections of rat's brain showed normal appearance (100%) as shown in table (2) and figure (10). Statistically, there was significant difference between control group and sham group, and the score of the control group showed that (66.6%) of the group had severe cerebral injury , (16.7%) had moderate cerebral injury and (16.7%) had slight cerebral injury as shown in table (2) and figure (10). Statistically, there was insignificant difference between control group and vehicle control group, and the score of the control vehicle group showed that (33.3%) had severe cerebral injury, (50%) had moderate cerebral injury and (16.7%) had slight cerebral injury as shown in table (2) and figure (10). pretreatment of rats with valsartan improved cerebral injury score significantly as compared with control vehicle group and the score of this group showed that (16.7%) had normal histopathological appearance, (66.6%) had slight cerebral injury and (16.7%) had moderate injury as shown in table (2) and figure (10 and 11). The histopathological cerebral changes are shown in figures (11-16).

Table 2: The differences in histopathological grading
of abnormal cerebral changes among the four
experimental groups.

Groups								
Valsar	tan	Contr vehic	ol le	Conti	rol	Sham		Histopathologicl Score
%	Ν	%	Ν	%	N	%	N	
16.7	1	0	0	0	0	100	6	Score 0(normal)
66.6	4	16.7	1	16.7	1	0	0	Score 1(slight)
16.7	1	50	3	16.7	1	0	0	Score2(moderate)
0	0	33.3	2	66.6	4	0	0	Score 3(sever)
100	6	100	6	100	6	100	6	Total



Figure 10: Component bar chart shows the relative frequency of different histopathological grading of abnormal cerebral changes among the four experimental groups.



Figure 11: A Photomicrograph of normal rat's brain section shows normal tissue and the histopathological score =0. The section stained with H&E (X 40).



Figure 12: Photomicrograph of rat's brain section after global cerebral ischemia shows edema(black

arrow) and the histopathological score = 1 (Slight injury). The section stained with H&E (X 40).



Figure 13: Photomicrograph of rat's brain section after global cerebral ischemia shows edema(black arrow) and hemorrhage(red arrow). The histopathological score = 2 (Moderate injury). The section stained with H&E (X 40).



Figure 14: Photomicrograph of rat's brain section after global cerebral ischemia shows edema(black arrow) and neutrophil infiltration(blue arrow). The histopathological score = 2 (Moderate injury). The section stained with H&E (X 40).



Figure 15: Photomicrograph of rat's brain section after global cerebral ischemia shows edema(black arrow), hemorrhage(red arrow) and area of necrosis and destructed neuron(green arrow). The histopathological score = 3 (Moderate injury). The section stained with H&E (X 40).



Figure 16: Photomicrograph for brain tissue of rats treated with valsartan drug shows mild edema (slight injury). The histopathological score =1 .The section stained with H&E (X 40).

Discussion

Effect of global cerebral ischemia reperfusion injury on inflammatory mediators (TNF- α , IL-6, IL-10)

In the present study, a significant increase (P < 0.05) in the inflammatory cytokine (TNF- α , IL-6 and IL-10) level was found in the control group as compared with the sham group. Chu et al. (2012)²⁰ showed that transient global cerebral IRI resulted in a substantial increase in the mRNA expression levels of TNF- α and IL-6 in the rat hippocampus. Jing et al. (2012) ²¹ data indicate that inflammatory response was initiated after transient cerebral ischemia and the release of inflammatory cytokines such as IL-6 and TNF- α occurred in the brain. Higher IL-6 levels have been detected in the peripheral blood of patients with acute cerebral ischemia than in control subjects ^{22,23}. Increased plasma and cerebrospinal fluid IL-6 levels are correlated with a larger infarct size ²². Zingarelli et al.(2001) ²⁴ established that the anti-inflammatory properties of endogenous IL-10 include negative modulation of secretion of proinflammatory TNF- α and IL-6, endothelial expression of P-selectin and ICAM-1, with consequent reduction of neutrophil infiltration and related oxidative and nitrosative stress. Zhai et al.(1997) 25 demonstrated that IL-10 and TNF- α gene expression is induced early following MCAO, where TNF- α induces IL-10, subsequently IL-10 inhibits TNF- α expression.

Effect of global cerebral ischemia reperfusion injury on apoptotic markers (caspase-3 and Bax)

In the present study, a significant increase (P < 0.05) in cerebral levels of Caspase-3 and Bax were found in the control group as compared with the sham group. Caspase-3 has been identified as a key mediator of apoptosis in animal models of ischemic stroke. Asahi et al.(1997) ²⁶ demonstrated upregulation of caspase-3 mRNA in rat brain 1 hr after the onset of focal ischemia. In addition, Namura et al.(1998) ²⁷ detected caspase-3 and its cleavage products in mouse brain during early reperfusion after 2 hr MCAO. Importantly, comparable observations have been extended to ischemic human brain tissue in that caspase-3 was upregulated after ischemia ²⁸. Liu et al.(2013) ²⁹ concluded that cerebral IRI may cause neurological impairment and causes neuron apoptosis that may be associated with the activation of caspase-3 and Bax and the down regulation of Bcl-2. Bax has been demonstrated to promote apoptosis, whereas Bcl-2 is important for cell survival and antiapoptotic effects ³⁰. Liu and Yang. (2004) ³¹ established that the expression of Bax mRNA in ischemic group was increased significantly as compared with sham group. Yin et al.(2013) ³² showed that the pro-apoptotic protein Bax content in brain tissues of ischemic reperfused group was markedly elevated as compared with sham-operated animals.

Effect of global cerebral ischemia reperfusion injury on oxidative stress markers (MDA and GSH)

In the current study, a significant increase (P < 0.05) in cerebral level of MDA was found in the control group as compared with the sham group; while there is a significant decrease (P < 0.05) in cerebral level of GSH for the control group as compared with sham group. It is well documented that transient global cerebral ischemia results in neurological abnormality and BCCAO for 30 min followed by 45 min of reperfusion was associated with increase generation of ROS and free radicals (Raghvendra et al., 2009) ³³. In agreement with our observation, Vekaria et al.(2012) ³⁴ found that BCCAO for 30 min, followed by

reperfusion for 45 min showed increase in MDA in brain affected by ischemic-reperfusion injury in controlled group which suggested enhanced lipid peroxidation. Cosar et al.(2014) ³⁵ demonstrated that when cerebral ischemia was performed via the occlusion of bilateral internal carotid artery for 45 minutes and continued with reperfusion process ,the MDA levels increased from sham group to IR group and the GSH levels decreased from sham to IR group. In the study of Vekaria et al.(2012) ³⁴, they observed that GSH levels decreased in hippocampus of ischemic rats (BCCAO control group) as compared to sham operated group. It has been shown that depletion in GSH levels in IRI can be attributed to several factors such as cleavage GSH levels to cysteine, decrease in synthesis of GSH and formation of mixed disulfides, causing their cellular stores to be depleted ³⁶. Also Mukherjee et al.(2007) 37 and Cosar et al.(2014) 35 observed that the GSH levels decrease due to cerebral IRI.

Effect of global cerebral ischemia reperfusion injury on T-Lymphocytes

In the current study, a significant increase (P < 0.05) in cerebral level of CD4⁺ T- lymphocyte was found in the control group as compared with the sham group, but there was insignificant changes in cerebral level of CD8⁺ T- lymphocyte in the control group as compared with the sham group. T lymphocytes are central to the development of a sustained inflammatory response and there is now good evidence that these cells accumulate in the post ischemic brain within a few hr of reperfusion ³⁸. Liesz et al. (2011b) ³⁹ also reported significant reduced infarct volumes in mice depleted of T helper and T cytotoxic cells following permanent ischemia. Thus, these findings suggest that both T helper and T cytotoxic cells contribute to the development of brain injury following stroke. Alison .(2006) 40 study strongly implicated both T lymphocytes and IFN- γ as key participants in the microvascular dysfunction and tissue injury that result from transient focal ischemia and reperfusion of mouse brain. Lai et al.(2007) 41 and

Winerdal et al.(2012) ⁴² demonstrated that the initial influx of T-lymphocytes was dominated by CD4+ Thelper cells, followed one week later by CD8⁺ cytotoxic T-cells. These are mainly in agreement with our results, where a significant increase in cerebral level of CD4⁺ T- lymphocyte was found in the control group, but there was insignificant changes in cerebral level of CD8+T- lymphocyte. The differences seen could be explained by the time of ischemia and reperfusion, where in our experiment, the time of ischemia was 30 min and the time of reperfusion was 1 hr.

Effect of global cerebral ischemia reperfusion injury on MPO-ANCA IgG

In the current study, a significant increase (P < 0.05) in cerebral level of MPO-ANCA IgG was found in the control group as compared with the sham group. MPO activity, which is an essential enzyme for normal neutrophil function that is released as a response to various stimulations 43, was evaluated by Cosar et al.(2014) ³⁵ who found that cerebral ischemia via the occlusion of bilateral internal carotid artery for 45 minutes and followed by reperfusion process, caused the MPO levels to increase from sham group to ischemic reperfused group. After MCAO for 2 hr, Chen et al.(2012) ⁴⁴ measured MPO activity at 6 and 24 hr of reperfusion, and found that neutrophil infiltration was significantly higher in the ischemic reperfused group than in the sham group. Annapurna et al.(2013) ⁴⁵ demonstrated that MPO activity was increased significantly in control vehicle group when compared to sham group and was correlated positively with infarct size.

Effect of global cerebral ischemia reperfusion injury on cerebral histopathology

There was a statistically significant difference between control group and sham group. The score of the control group shows slight and moderate cerebral injury. From the histopathological study of Prakash et al.(2011) ⁴⁶, it was observed that sections of brain tissue showed swollen neurons, dilated blood vessels with neuronal loss occurred in brain regions of

ischemic reperfused rats induced by BCCAO for 30 min followed by 1 hr and 4 hr reperfusion in ischemic control group . While no apparent morphological changes in sham and brain section showing normal 17 structure. Chandrashekhar et al.(2010) demonstrated that global cerebral ischemia on rats by BCCAO for 30 min followed by 1 hr reperfusion caused marked congestion of blood vessels and neutrophil infiltration and neuronal necrosis. Shah et al. (2005) ⁴⁷ found that in BCCAO for 30 min, caused marked congestion of blood vessels and these effects were further augmented following reperfusion for 1hr i.e. lymphocytic proliferation and neuronal necrosis.

Effect of valsartan on study parameters

Effect of valsartan on inflammatory markers (TNF α , IL-6 and IL-10)

The present study showed that valsartan administration before the induction of cerebral ischemia caused a significant lowering (P<0.05) in cerebral level of TNF- α , IL-6 and a significant increase (P<0.05) in IL-10 as compared with control and control vehicle group. So our results indicated that valsartan, an ARB, can prevent cerebral inflammation and decrease ischemic brain damage. This finding is in consent with Saavedra et al. (2006) ⁴⁸ who strongly suggested that inhibition of AT(1) receptors considered as a preventive therapeutic measure to protect the brain from ischemia, and as a possible novel therapy of inflammatory conditions of the brain. Wu et al(2001) ⁴⁹ showed that valsartan attenuated the expression of MCP-1, TNF- α , IL-6, IL-1 β , and infiltration of leukocytes and macrophages in the injured arteries; these results suggested that the stimulation of the AT(2) receptor after AT(1) blockade is important in the improvement of the inflammatory vascular injury. Kikuchi et al.(2012) 50 stated that cerebral inflammation was also prevented by valsartan, and this was associated with suppression of inflammatory cytokines such as MCP-1 and TNF- α by valsartan. Peeters et al.(1998) ⁵¹ found that the AT(1) receptors antagonist, valsartan, has potent inhibitory effects on the lipopolysaccharide(LPS)-stimulated

production of inflammatory cytokines, TNF- α and IL-6 in vitro. Santiago et al.(2008) ⁵² suggested a possible anti-inflammatory effect for valsartan in colitis via modulation of the immune system. In their study valsartan treatment caused an upregulation in the gene expression of IL-10, a cytokine which is known to be therapeutic in colitis, confirmed by an increase in IL-10 positive cells staining by immunohistochemistry in the valsartan treated animals. To the best of our knowledge, there is no data available about the effect of valsartan on IL-10 in global cerebral IRI.

Effect of valsartan on apoptotic markers (caspase-3 and Bax)

The current study showed that valsartan administration before the induction of cerebral ischemia caused a significant decrease (P<0.05) in cerebral level of Caspase-3and Bax as compared with control and control vehicle group . So our results indicated that valsartan can reduce cerebral apoptosis and decrease ischemic brain injury. Kikuchi et al.(2012) ⁵⁰ stated that valsartan prevented neuronal apoptosis, and this was associated with the suppression of apoptosis signal-regulating kinase 1 activation by valsartan. Tang et al.(2013) ⁵³ found that in the primary cultured neurons, in which the in vitro ischemic reperfused model was established, Ang II increased the ratio of bcl2/bax in mRNA expression and valsartan was shown to inhibit cell apoptosis. Experimental and clinical studies have revealed that ARB has protective effects against ischemic brain injury, but the mechanism is still obscure. ARB may also have effects on neurogenesis through the activation of unblocked AT II type 2 receptors⁵⁴. Wakai et al.(2011) ⁵⁴ showed that Val significantly suppressed superoxide production and cytochrome C release into the cytosol after transient forebrain ischemia and consequently attenuated ischemic neuronal damage without affecting the blood pressure in rats. Their results suggest that valsarta has neuroprotective effects on ischemic injury through the suppression of oxidative stress and mitochondrial injury.

Effect of valsartan on oxidative stress markers (MDA and GSH)

The current study showed that valsartan administration before the induction of cerebral ischemia caused a significant lowering (P<0.05) in cerebral level of MDA and a significant increase (P<0.05) in GSH as compared with control and control vehicle group. So our results indicated that valsartan can attenuate oxidative damage of the brain. This finding is in agreement with Yang et al.(2014) 55 who showed that valsartan significantly restored SOD and GSH activities and reduced MDA level in cortex and hippocampus indicating attenuation of oxidative stress and attenuate oxidative damage. Li et al.(2008) ⁵⁶ established that MCAO increased superoxide production in the ischemic area of the brain, and temporary treatment with valsartan attenuated superoxide production, where as continuous treatment with valsartan decreased oxidative stress. Jiao et al.(2011) ⁵⁷ revealed that valsartan exhibited renoprotective effects through restoring the levels of oxidative stress relevant molecules (GSH,SOD and MDA), results suggested that the renoprotective effects of valsartan may be related to its potential in decreasing oxidative stress . Qin et al.(2011) 58 showed that in rats with vascular dementia induced by BCCAO, valsartan increased the activities of SOD and GSH and decreased MDA activities in hippocampal tissues. The damages in structure, number and volume of hippocampal neuron cells were reduced by valsartan.

Effect of valsartan on T-Lymphocytes

The current study showed that valsartan administration before the induction of cerebral ischemia caused a significant changes (P<0.05) in cerebral level of CD4+ T-Lymphocytes and insignificant changes in cerebral level of CD8+ T-Lymphocytes as compared with control and control vehicle group. This effect of valsartan on Tlymphocyte could be due to its pleotropic effect as anti-inflammatory, anti-oxidant and anti-apoptotic agent. To the best of our knowledge, there is no data available about the effect of valsartan on CD4⁺ and CD8⁺ T-Lymphocytes in global cerebral IRI.

Effect of valsartan on MPO-ANCA IgG

The current study showed that valsartan administration before the induction of cerebral ischemia caused a significant lowering (P<0.05) in cerebral level of MPO as compared with control and control vehicle group. This could be due to its anti-inflammatory effect via suppression of inflammatory cytokines such as MCP-1 andTNF- α ^{59,50}. To the best of our knowledge, there is no data available about effect of Val on MPO on global cerebral IRI.

Effect of valsartan on brain histopathology

In the current study, pretreatment with valsartan for 10 days before cerebral ischemia ameliorated the brain injury significantly as compared with control group. Li and Zhou. (2010) ⁶⁰ found that the cerebral neuron damage of spontaneously hypertensive rats whose ultrastructure showed cell-pyknosis, chromatin margination and typical apoptotic body formation were alleviated after the intervention of valsartan. Qin et al.(2011) ⁵⁸ showed that the damages in structure, number and volume of hippocampal neuron cells were reduced by valsartan.

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