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Effect of Angiotensin Receptor Blokers (ARBs) and Calcium Channel Blockers (CCBs) In Rat Cerebral Ischemia Reperfusion I/R Injury.

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ABSTRACT

This study was undertaken to investigate the possible neuroprotective activity of candesartan, amlodipine and their combination in amelioration of global cerebral I/R injury in rat model. Adult Sprague dawley rats were randomized in to 6 groups as follow: group I, sham group, rats underwent the same anesthesia and surgical procedure as the control group with out bilateral common carotid artery occlusion (BCCAO) ; group 2 control group (induced-untreated), rats underwent 30 min of global cerebral ischemia via (bilateral common carotid artery occlusion (BCCAO) followed by 1 hour of reperfusion ; group 3, Control – Vehicle, as control group but rats received daily for 10 days before the surgery the vehicle of amlodipine and candesartan drugs normal saline intraperitoneally (IP),the dose of vehicle was (0.9% Nacl) ,(1 ml/kg/day) ; group 4, amlodipine treated group, as control group, but rats received daily amlodipine intraperitoneally (IP), the dose of amlodipine was (10 mg/kg /day) for 10 days before the surgery ; and group5, candesartan treated group, as control goup ,but rats received daily candesartan intraperitoneally (IP), the dose of candesartan was (0.2 mg/kg /day) for 10 days before the surgery ; group 6, combination candesartan and amlodipine treated group, as control group, but rats received daily intraperitoneally (IP) combination of candesartan (0.2 mg/kg /day) and amlodipine (10 mg/kg /day) for 10 days before the surgery. Compared with the sham group, levels of cerebral IL-9, IL-6, MCP-1 and ICAM-1, increased significantly ($p<0.05$), both candesartan, amlodipine, and their combination significantly oppose the increase in cerebral level of IL-9, IL-6, MCP-1 and ICAM-1 ($P < 0.05$). Histological analysis revealed that each of candesartan, amlodipine, and their combination markedly reduced ($P < 0.05$) the severity of brain injury in the rats underwent bilateral common carotid artery occlusion (BCCAO), and infarct size significantly reduction ($P < 0.05$).The results of the present study reveal that pretreatment with candesartan amlodipine, and their combination may ameliorate the global cerebral ischemia-reperfusion injury by anti-inflammatory effect and the combination of candesartan and amlodipine show edasynergistic effect in protection against brain ischemia.

Keywords: Global cerebral ischemia, inflammation, candesartan, amlodipine.

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INTRODUCTION

Acute ischemic stroke is the second leading cause of death in the world.In the Middle East and North Africa stroke is increasingly becoming a major health problem, with projections that deaths from it will nearly double by 2030 [1]. Three months following a stroke, 15-30% of stroke survivors are permanently disabled and 20% require institutional care [2].Deficits can include partial paralysis, difficulties with memory,thinking, language, and movements. Brain ischemia occurs when cerebral blood flow is reduced to a low level by certain pathological conditions, such as stroke or cardiac arrest [3,4,5].Global cerebral ischemia occurs commonly in patients who have a variety of clinical conditions including cardiac arrest (CA), shock, and asphyxia and in patients undergoing complex cardiac surgery [6,7,8].Cerebral ischemia and reperfusion initiates a complex cascade of pathological events, include excitotoxicity, peri-infarct depolarizations, inflammation and programmed cell death[9].The cellular changes caused by a reduction in blood flow (i.e., the primary injury) include a reduction in oxygen delivery, a switch to anaerobic glycolysis, a progressive fall in high-energy phosphate compound i.e., adenosine triphosphate (ATP) , intracellular acidosis, and intracellular accumulation of sodium and calcium ,and loss of cell ion homeostasis , generation of arachidonic acid products, cytokine mediated cytotoxicity, activation of glial cells [10].Rapid reperfusion, although intended, contributes to secondary injury by a cascade of pathological processes including leukocyte infiltration, platelet and complement activation, postischemichyperperfusion , hemodynamic disturbances, inflammatory processes, free radical formation and breakdown of the blood-brain barrier (BBB) [11,12] . When severely impaired, there is an increased risk of deleterious vasogenic edema, brain herniation and death 12. Ischemia and reperfusion in the brain, as in other organs, induces an inflammatory response which may exacerbate initial levels of tissue injury [13]. Inflammation is characterized by the accumulation of inflammatory cells and mediators in the ischemic brain. In the acute phase (minutes to hours) of ischemic stroke, ROS and proinflammatory mediators (cytokines and chemokines) are released rapidly from injured tissue[14,15]

Interleukin-6 is generally considered to be a pro-inflammatory cytokine.IL-6 expression significantly increases in the acute phase of cerebral ischemia [16,17] .In patients with acute brain ischemia, plasma concentrations of IL-6 are strongly associated with stroke severity and long-term clinical outcome [18,19,20] . Increased plasma and cerebrospinal fluid IL-6 levels are correlated with a larger infarct size on CT and magnetic resonance imaging scans [21,22] and with a poorer clinical outcome [23]. Interleukin-9 (IL-9) is a multifunctional cytokine produced by activated TH2 clones in vitro and during TH2-like T cell responses in vivo [24]. And, Ormstad et al. (2011) [25] showed that a significant elevation in IL-9 in the acute ischemic stroke(AIS group). And the findings of elevated levels of IL-9 in acute ischemic stroke AIS patients are novel.Chemokine that has been associated with ischemia/reperfusion injury is chemoattractant protein-1 (MCP-1).The MCP-1 levels are increased in the cerebrospinal fluid of stroke patients [26]. Expression of chemokines following focal ischemia is thought to have a deleterious role by increasing leukocyte infiltration [27].Consistent with a deleterious role, their inhibition or deficiency is associated with reduced injury [28].And there is increasing evidence that cellular adhesion molecules (CAMs) play an important role in the pathophysiology of acute ischemic stroke[29].At the clinical level, increased sICAM-1 and sVCAM-1 have been documented in the plasma and cerebral spinal fluid of subjects with recent cerebral ischemic patients, and correlated to stroke severity [30,31,32]

Amlodipine, a third generation dihydropyridine calcium antagonist, is characterized by a higher vascular selectivity and a smaller negative inotropic effect compared to Nifedipine[33].Dihydropyridine calcium-channelblockers are nowbeing used to treat several disorders, such as hypertension,arrhythmia, angina pectoris, left ventricular diastolic dysfunction,myocardial infarction, Raynaud's phenomenon, andprogressive systemic sclerosis[34,35].Amlodipine, besides being a Ca2+ channel blocker, has also antiinflammatory-antioxidant and antiapoptotic activity [36,37].Umemoto et al. (2004)[38] showed in a stroke model of hypertensive rats treated with amlodipine, brain tissue damage was low,and this effect was suggested to be associated with the increasing effect of amlodipine on superoxide dismutase (SOD) activity.That confirmed by Mogi et al. (2006)[39] who suggested that amlodipine treatment was reduces stroke size and neurologic deficit after focal brain ischemia, possibly through an increase in cerebral blood flow and inhibition of superoxide production .

Candesartan is an angiotensin II receptor blocker (ARB). ARBs are widely used in treatment of diseases like hypertension, heart failure, myocardial infarction and diabetic nephropathy[40].Hosomi et al. (2005) ; Engelhorn et al. (2004) ; Takagi et al. (2006) ; Araya et al. (2002) [41,42,43,44] these studies showed that

candesartan, a selective AT1 receptor antagonist, may reduce cerebral I/R injury.Ando et al. (2004)[45] showed that the antiinflammatory effects of AT1 receptor antagonists may be an important mechanism in protecting against ischemia.One possible mechanism by which the AT1-receptor blocker might reduce infarct size in the reperfusion phase after cerebral ischemia is through inhibition of inducible nitric oxide synthase (iNOS) expression. Nitric oxide produced by iNOS is one of the factors contributing to the expansion of brain damage in the late postischemic period [46].

MATERIALS AND METHODS

Animals:

A total of 36Adult Sprague-Dawley rats weighing (150-220 g) were obtained from Animal Resource Center, the National Center for Drug Control and Researches. The animals were apparently healthy and they were housed in the animal house of College of Medicine/ University of Kufa, at temperature controlled environment (25±2°C) with ambient humidity. Lights were maintained on a 12 h light/dark cycle. The rats received standard chow diet with water. Rats in the study were maintained in accordance with the guidelines established by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

Preparation of Candesartan and amlodipine:

Candesartan andamlodipinewere provided from (Pioneer co. Sulaymanieyah/Kurdistan Iraq),Candesartan and amlodipine were prepared immediately before use by dissolving them innormal saline.

Experimental Groups:

After one week of acclimatization, the rat were randomized into four groups (6 rats in each group) group 1,sham group rats underwent the same anesthetic and surgical procedures for an identical period of time ,but with out bilateral common carotid artery occlusion (BCCAO) ; group 2,Control group (induced untreated) rats underwent anesthesia and surgery with bilateral common carotid artery occlusion (BCCAO) for 30 min. and then reperfusion for 1 hour But without drugs ; group 3,Control - Vehicle group for 10 days before surgery rats received daily intraperitoneally (IP) the vehicle of candesartan and amlodipine normal saline (0.9% Nacl) (1 ml/kg/day) [47,48].Then, anesthesia and surgery with bilateral common carotid artery occlusion (BCCAO) for 30 min. and later reperfusion for 1 hour.

group 4, Candesartan treated group rats received daily candesartan intraperitoneally (IP) . The dose of candesartan was (0.2 mg/kg /day) [47], for 10 days before the surgery ,then anesthesia and surgery with bilateral common carotid artery occlusion (BCCAO) for 30 min. and later reperfusion for 1 hour ; group 5, Amlodipine treated group rats received daily amlodipine intraperitoneally (IP) . The dose of amlodipine was (10 mg/kg /day) [49] for 10 days before the surgery ,then anesthesia and surgery with bilateral common carotid artery occlusion (BCCAO) for 30 min. and later reperfusion for 1 hour. ; group 6, combination of candesartan and amlodipine treated group, as control group, but rats received daily intraperitoneally (IP) combination of candesartan (0.2 mg/kg /day)[47]and amlodipine (10 mg/kg /day)[49] for 10 days before the surgery.

Induction of global brain ischemia:

Induction of global ischemia by bilateral common carotid artery occlusion (BCCO)[**50,51]** rats were maintened at approx 37°C under a light bulb and under general anesthesia ketamine &xylazine(80mg/kg&5mg/kg intraperitoneally) [**52].**Animals were placed on the back in the supine position **.**A small median incision was made in the neck and both carotid arteries were separated from vagal nerves, then exposed bilaterally and occluded by using atraumaticmicroclamps and clamped for 30 min.In the reperfusion, the clamp were removed after ischemia and reperfusion was allowed to take place for 1 hour.

Preparation of Samples:

Tissue Preparation for IL-6,IL-9,ICAM-1and MCP-1 Measurement:

Following decapitation, the brain was removed and washed in cooled 0.9% saline, kept on ice and subsequently blotted on filter paper, then weighed and homogenised using a high intensity ultrasonic liquid processor and brain tissues were homogenized in ice-cold 1:10 (w/v) 0.1 M phosphate-buffered saline (PBS) (pH 7.4), containing protease inhibitor cocktail and 0.2% Triton X-100 for 30 seconds[53] . The homogenates were centrifuged at 14,000×g for 20 min at 4◦ C and the supernatant was collected for determination of IL-6,IL-9,ICAM-1and MCP-1 according to the manufacturer's instructions and guidelines using enzyme-linked immunosorbent assay (ELISA) kits (Quantikine®/R&D Systems.USA and Uscn. Life Science Inc. ,USA).

Tissue Sampling for Histopathology:

Coronal brain sections from control and experimental groups of global ischemia were fixed with 10% formalin and embedded in paraffin wax the sections were stained with haemotoxylin and eosin dye (H&E) for histopathological observation[**54].**The histological observations (evaluated by a pathologist using a double-blind method) were scored using a pathological scoring scale as follows: 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 and 3,(severe) = clearly infarctive foci (local necrosis).

Measurement of infarction area:

Rats were sacrificed after 30min of BCCAO and 1 hour of reperfusion tissue damage or the infarction area was measured by 2,3,5-triphenyltetrazolium chloride(TTC) staining method according toBederson(1986)[**55] .**TTC solution was prepared by dissolved TTC 2% (W/V) in PBS (phosphate buffer saline) (pH 7.4-7.6) , with 37°C[**56].** The brain slices were put in a glass petri dish containing a shallow layer of TTC solution and incubated at 37°C for 30 minutes **.**The TTC solution was then replaced with 10% buffered formalin (phosphate-buffered formalin ,PBF)**.** The fixed brain sections were photographed and analysis by image analysis software (Digimizer) ,the unstained areas of the fixed brain sections were defined as infracted

Statistical Analysis:

Statistical analyses were performed by using SPSS 17.0 for windows lnc. An expert advice was consulted for tests used. Data were expressed as mean ± SEM. Analysis of Variance (ANOVA) was used for the multiple comparisons among all groups followed by post-hoc tests using LSD method.The histopathological brain changes are a non-normally distributed variable measured on an ordinal level of measurement; therefore non-parametric tests were used to assess the statistical significance involving this variable. The statistical significance of difference in total score between more than 2 groups was assessed by Kruskal-Wallis test, while Mann-Whitney U test was used for the difference between 2 groups. In all tests, $P < 0.05$ was considered to be statistically significant..

RESULTS

Effect on Proinflammatory markers (IL-6, IL-9, MCP-1 and ICAM-1):

At the end of the experiment, the levels of cerebral IL-6, IL-9, MCP-1 and ICAM-1 were significantly ($P<0.05$) increased in control group as compared with sham group. The levels of cerebral IL-6 ,IL-9, MCP-1 and ICAM-1 of candesartan,amlodipine and their combination treated group were significantly ($p<0.05$) lower than that of control-vehicle group. The values of cerebral IL-6 ,IL-9, MCP-1 and ICAM-1 are showed in figures 1, 2, 3 and 4.

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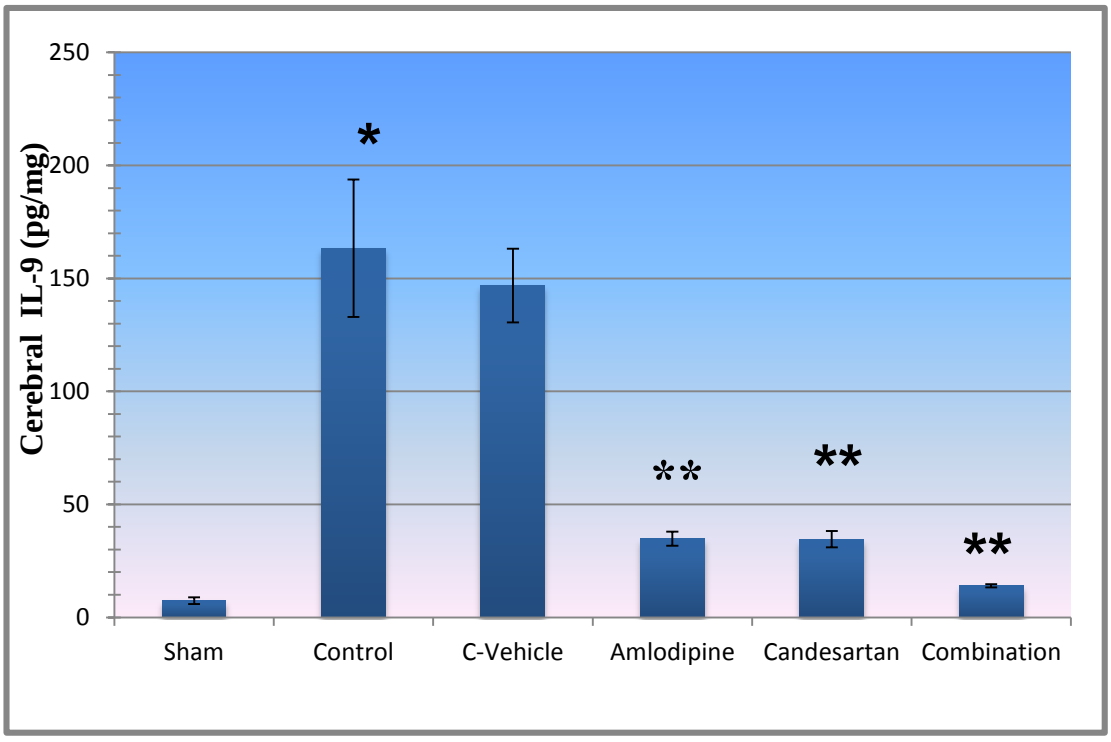

Figure (1): Error bar chart shows the difference in mean± SEM values of cerebral IL-9 level (pg/mg) in the six experimental groups at the end of the experiment (No. of animals = 6 in each group).* P < 0.05 vs. sham group, ** P < 0.05 vs. control-Vehicle group

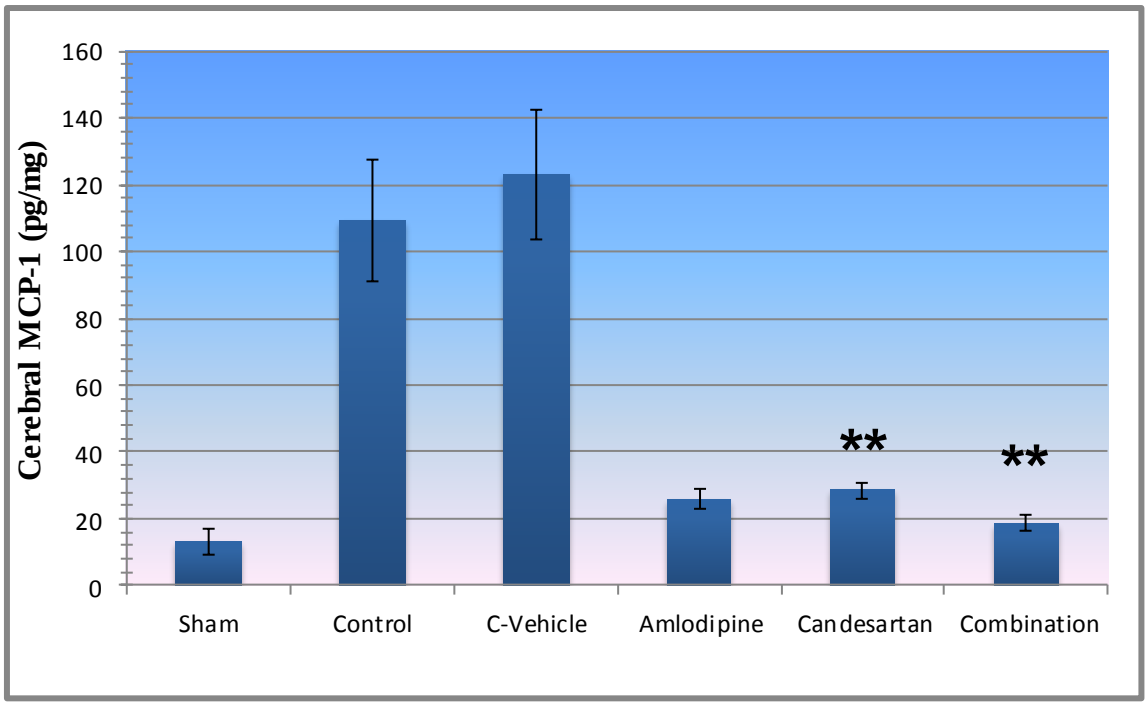

Figure (2): Error bar chart shows the difference in mean± SEM values of cerebral MCP-1 level (pg/mg) in the six experimental groups at the end of the experiment (No. of animals = 6 in each group).* P < 0.05 vs. sham group, ** P < 0.05 vs. control-Vehicle group.

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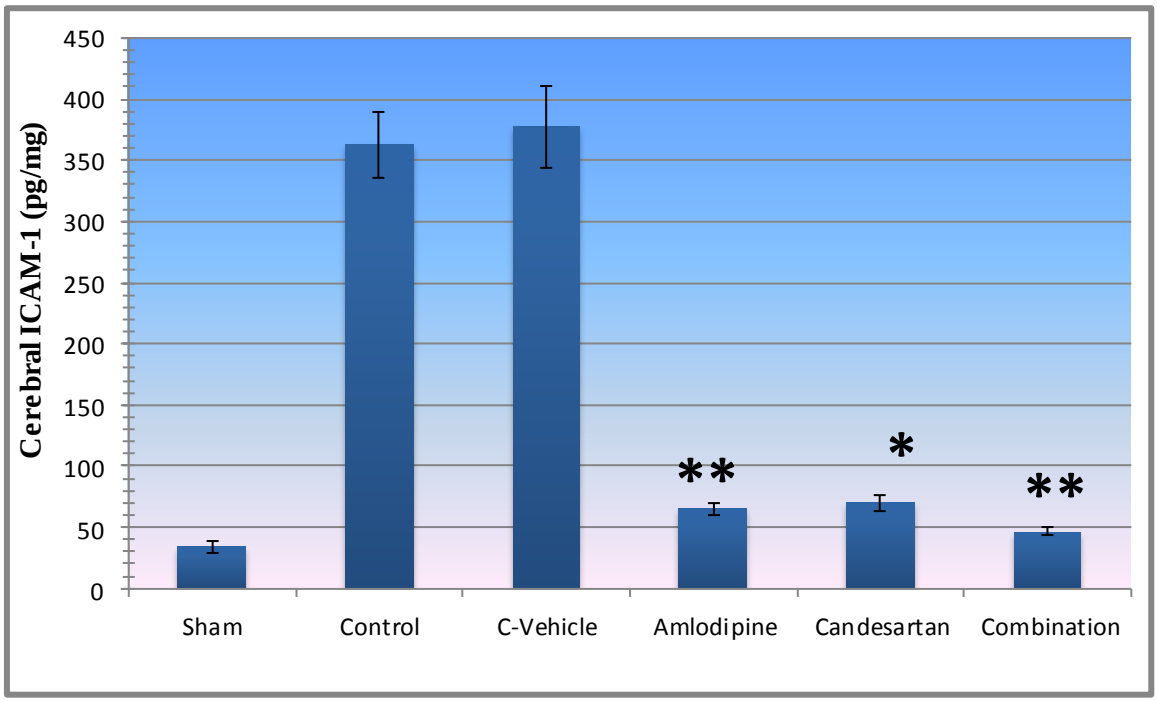

Figure (3): Error bar chart shows the difference in mean± SEM values of cerebral ICAM-1 level (pg/mg) in the six experimental groups at the end of the experiment (No. of animals = 6 in each group).* P < 0.05 vs. sham group, ** P < 0.05 vs. control-Vehicle group.

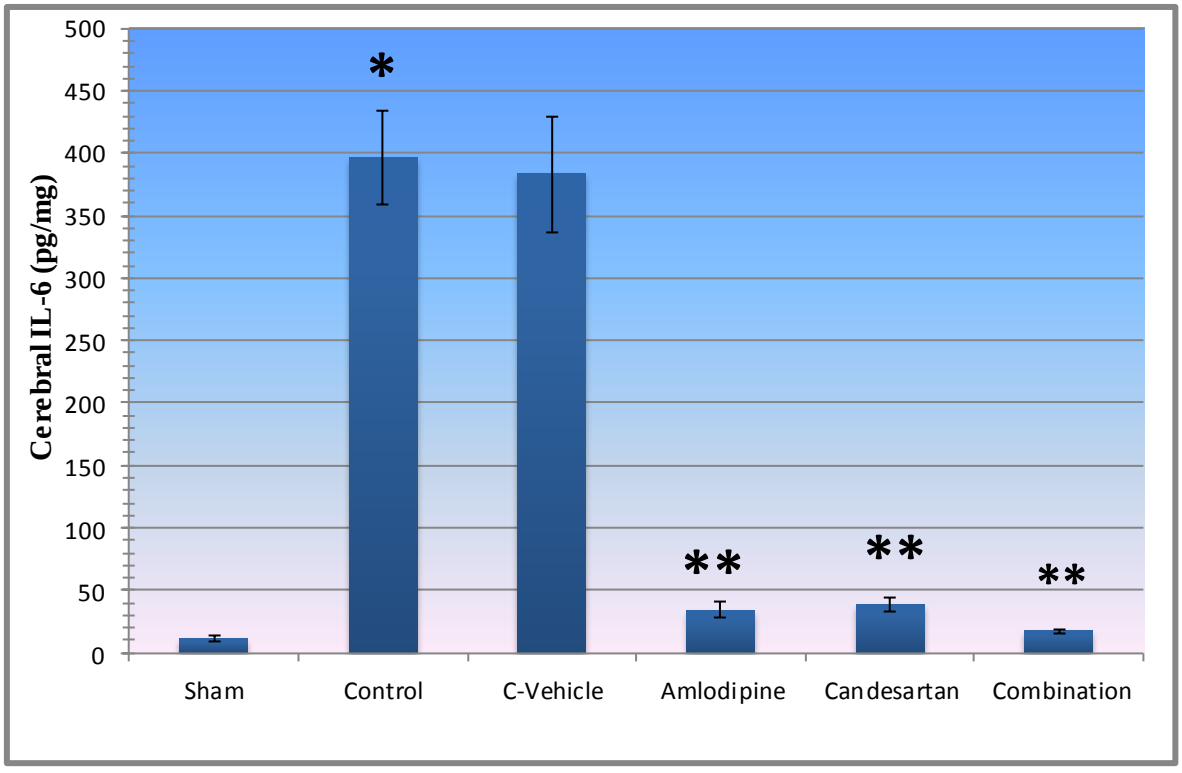

Figure (4): Error bar chart shows the difference in mean± SEM values of cerebral IL-6 level (pg/mg) in the six experimental groups at the end of the experiment (No. of animals = 6 in each group).* P < 0.05 vs. sham group, ** P < 0.05 vs. control-Vehicle group.

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Histopathological Findings:

A cross section of sham rat's brain showed a normal appearance (100%) of rats in this group and also showed normal brain appearance as shown in table(1)and figures(5,6)**.**There was statistically significant difference between control group (II) and sham group (I) ($P < 0.05$) and the total severity scores of the control group showed severe cerebral injury (66.6%)**.** And it (33%) showed moderate injury as shown in table(1) and figures (5,6)**.**Treatment of rats with amlodipine improved cerebral injury score significantly ($P < 0.05$) as compared with control – vehicle group and the total severity scores mean of this group showed (16.7%) of the group had normal histopathologicalappearance,and (66.6%) of the group had slight cerebral injury ,and (16.6%) had moderate injury as shown in table (1) figure (5,6). Treatment of rats with candesartan improved cerebral injury score significantly ($P < 0.05$) as compared with control – vehicle group and the total severity scores mean of this group showed (16.6%) had normal histopathological appearance and (50%) of the group had slight cerebral injury and (33.3%) had moderate injury as shown in table (1) figure (5,6). Treatment of rats with combination improved cerebral injury score significantly ($P < 0.05$) as compared with control – vehicle group and the score of this group were:(33.3%) had normal histopathological appearance and (66.6%) had slight injury as shown in table (1) figure (5,6).

Histopathologicl Score	Study groups											
	Sham		Control		Control-vehicle		Amlodipine		Candesartan		Combination	
	N	%	N	%	N	%	N	%	N	%	N	%
Normal (0)	6	100	0	0	0	0	1	16.7	1	16.7	2	33.3
Slight (1)	0	0	0	0	0	0	4	66.6	3	50	4	66.6
Moderate (2)	0	0	2	33.3	3	50	1	16.7	2	33.3	0	0
Severe (3)	0	0	4	66.6	3	50	0	0	0	0	0	0
Total	6	100	6	100	6	100	6	100	6	100	6	100

Table (1): The differences in histopathological grading of abnormal cerebral changes among the six experimental groups.

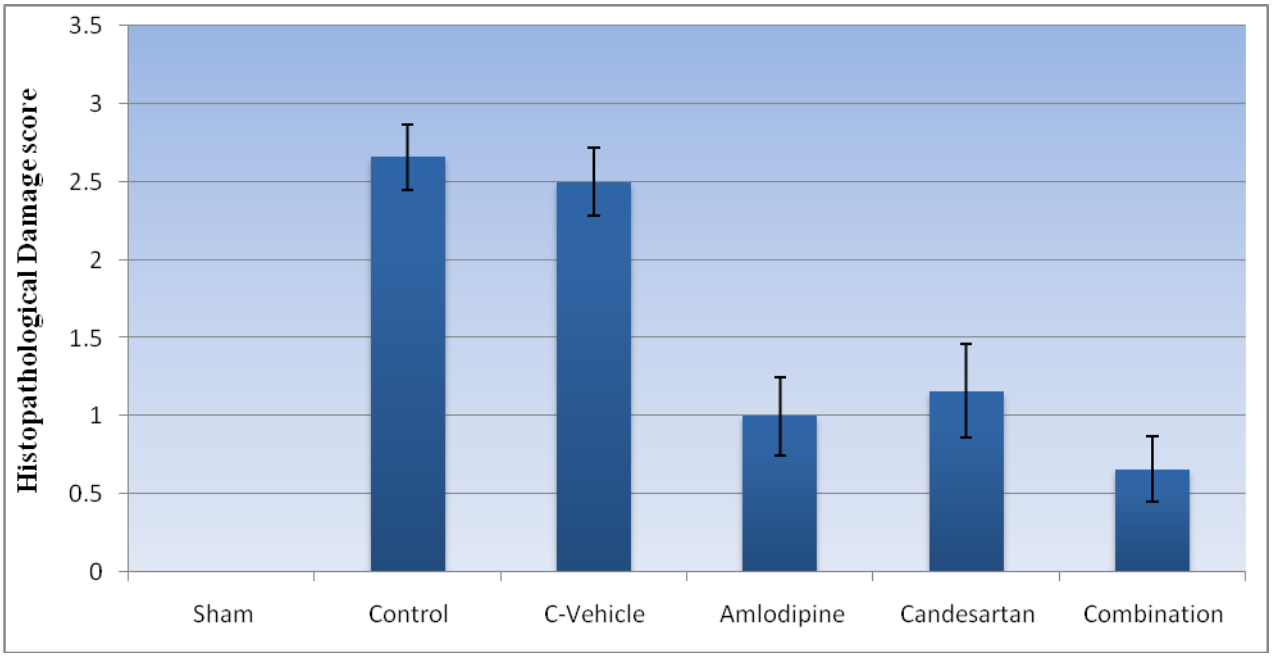

Figure (5): Error bar chart shows the difference in mean± SEM values of total severity scores in the six experimental groups at the end of the experiment (No. of animals = 6 in each group).

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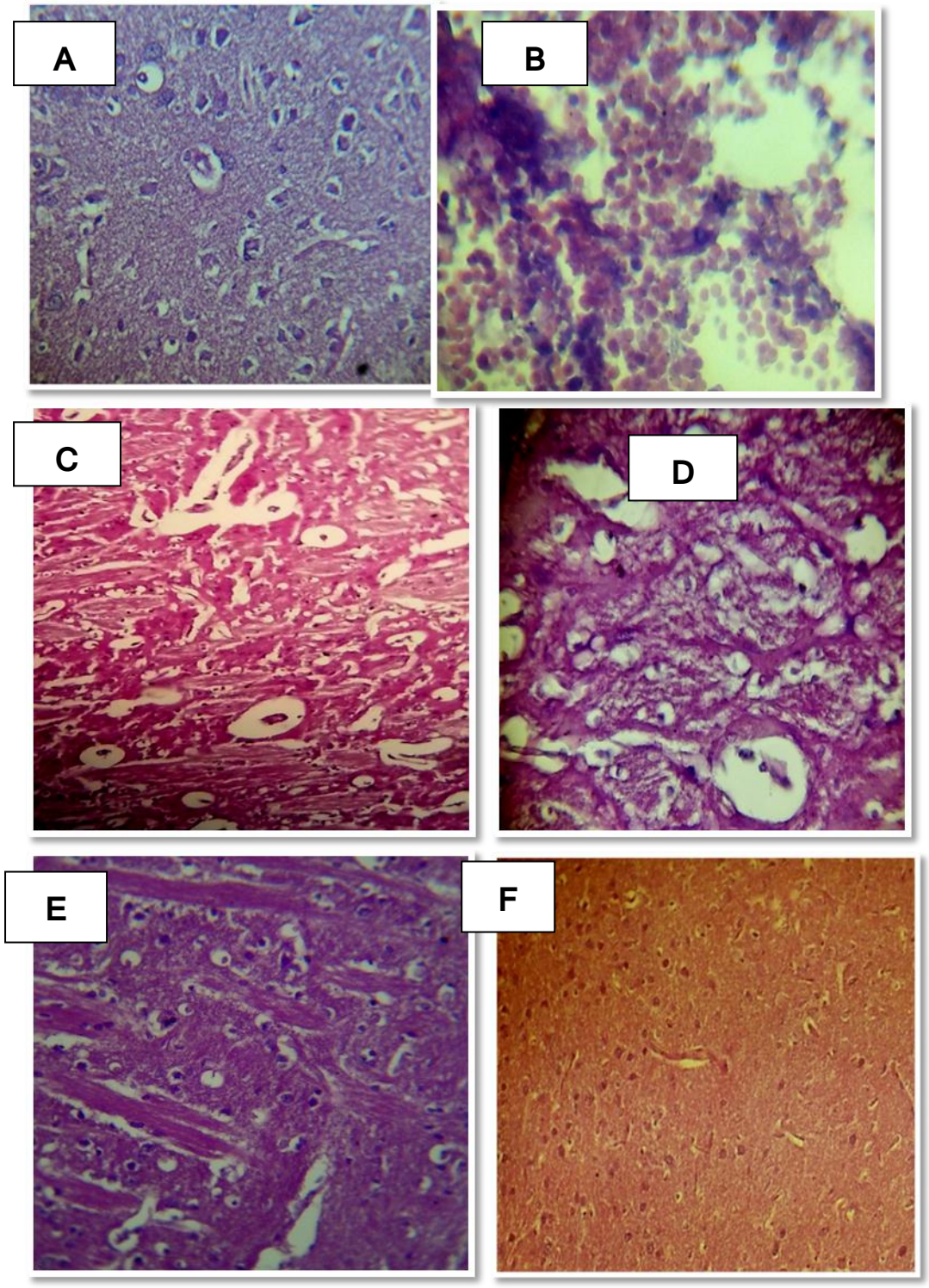

Figure (6): Photomicrograph represent the histopathological changes in rats. A: section of rat brain shows the normal architecture B: cerebral section with moderate injury showed cell infiltration;C: cerebral section with severe injury showed necrosis (infarct foci)& hemorrhage; D: cerebral section in amlodipine treated group showed slight injury (eosinophilic neurons); E: cerebral section in candesartan treated group showed slight injury (dark neurons ,pyknotic cells); F: cerebral section in Combination of (Amlodipine & Candesartan) drugs showing normal tissue (normal histological appearance).Sections stained with H&E (X40).

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Assessment of cerebral infarct size:

At the end of the study, the cerebral infarction area in the rat's brain of the six experimental groups was measured by 2,3,5-triphenyltetrazolium chloride (TTC) staining**.** It significantly ($p < 0.05$) increased in control group (II) as compared with sham group **.** There was insignificant difference between control – vehicle and control group.The cerebral infarct size in each of the Candesartan treated group, amlodipine and their combination treated group was significantly ($p<0.05$) lower than that of control –vehicle group. As compare with control group amlodipine treated group reduce infarct size (22.6%) ,and in Candesartan treated group reduce infarct size (22.9%) . while their combination reduced the cerebral infarction area in the rat's brain to (29.8%) .The changes in cerebral infarct size are summarized figure (7) .

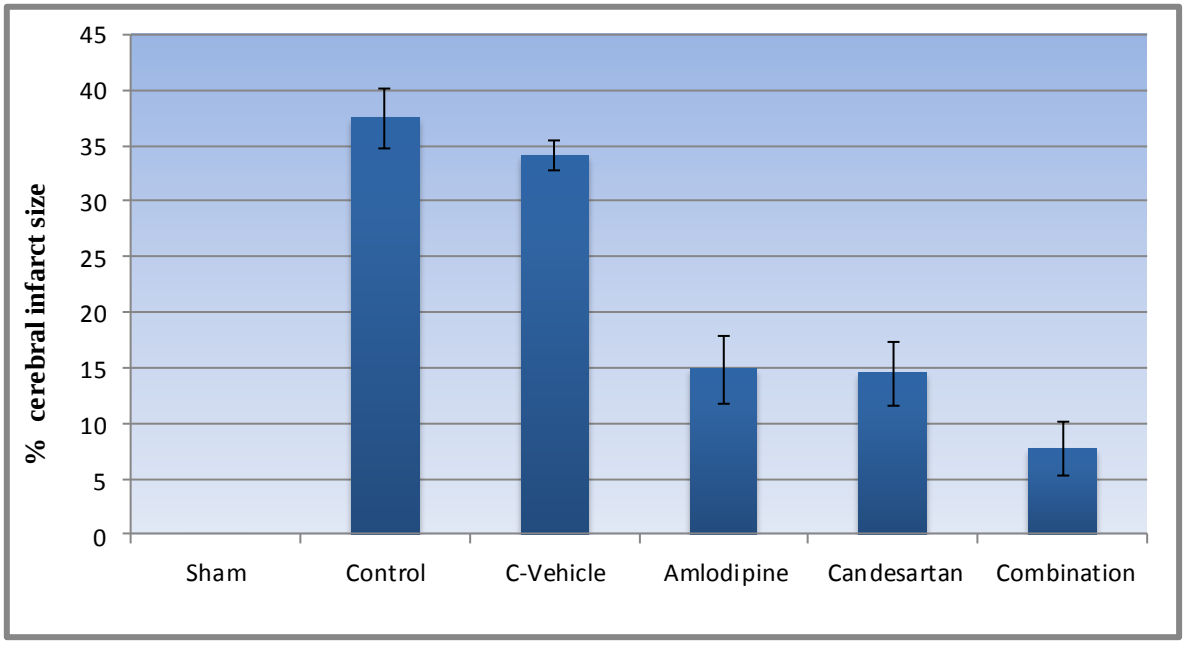

Figure (7): Error bar chart shows the difference in mean± SEM values of cerebral infarct area percent in the six experimental groups at the end of the experiment (No. of animals = 6 in each group).

DISCUSSION

Transient cerebral ischemia-reperfusion (IR) injury is a major complication in stroke, recovery, and perioperative period, in which 50–70% of survivors suffer from severe disabilities[57].Ischemia and reperfusion in the brain, as in other organs, induces an inflammatory response which may exacerbate initial levels of tissue injury[13].This inflammatory up-regulation occurs, anywhere from minutes to days after the ischemic event[58]

Effect of Global Cerebral Ischemia Reperfusion Injury on Inflammatory Mediator (IL-6):

In this study a significant increase in inflammatory mediator (IL-6) level in cerebral tissues ($p<0.05$) was found in control group as compared with sham group.Tarkowski et al. (1995) & Beamer et al. (1995)[21,22] were the first who demonstrated that increased plasma and cerebrospinal fluid IL-6 levels are correlated with a larger infarct size on CT and magnetic resonance imaging scansand with a poorer clinical outcome[23] . Becker et al. (1998)59 later demonstrated that inflammatory pathways can contribute to cell death following ischemia/reperfusion, i.e., secondary brain injury. The results in the present study are in agreement with those reported byWang et al. (1995b)[60] who found that Early (within 15 minutes) induction of mRNA for interleukin IL-6 has been shown in neurons (IL-6 in astrocytes) .

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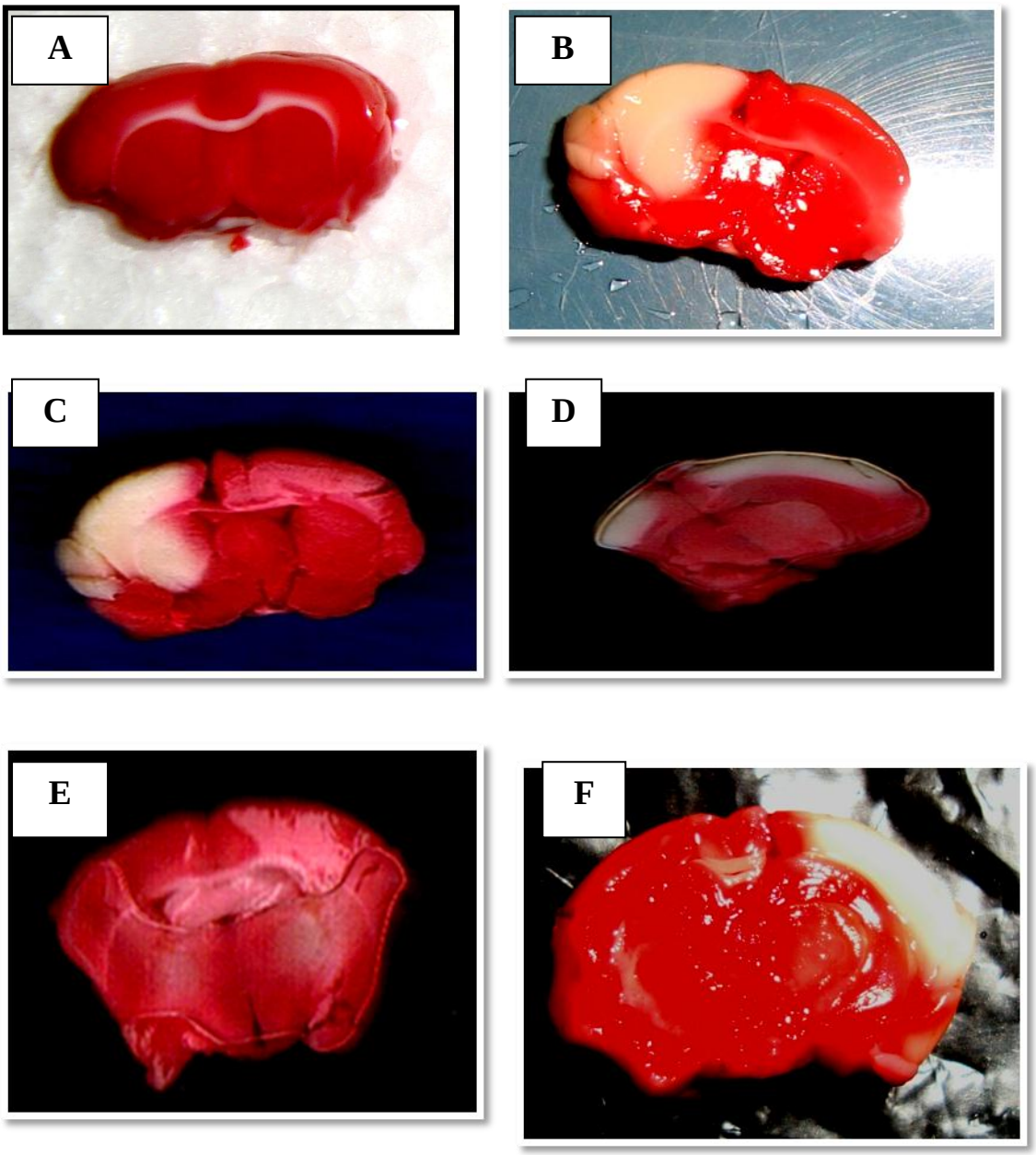

Figure (8) : Photograph of brain slides A: sham group stained by TTC stain showing normal brain no cerebral infarction ; B: control group showed significant increase in cerebral infarction area percent to (37%) ; C: control-vehicle group showed (32%) cerebral infarction area ; D: treated with amlodipine showed significant decrease in cerebral infarction area percent to (17%) ; E: treated with candesartan showed significant decrease in cerebral infarction area percent to (17%) ; F: treated with combination (amlodipine & candesartan) showed significant decrease in cerebral infarction area percent to 11 %.

Effect of Global Cerebral I/R on IL-9 inflammatory Cytokine:

In the present study a significant increase in inflammatory cytokine (IL-9) level ($P< 0.05$) was found in the I/R rats as compared with sham group.Patkai et al. (2001)[61] demonstrated that elevated mean IL-9 serum levels have been observed in human neonates who will later develop cerebral palsy.The results in the present study are in agreement with that reported byOrmstad et al. (2011)25 who found that a significant elevation in IL-9 in the acute ischemic stroke(AIS group), and the findings of elevated levels of IL-9 in acute ischemic stroke AIS patients are novel.

Effect of Global Cerebral I/R on Chemokine MCP-1:

In the present study, a significant increase in inflammatory chemokine (MCP-1) level (P< 0.05) was found in the I/R rats as compared with sham group. Amantea et al. (2009) and Losy et al. (2001)[14,62]demonstrated that MCP-1 levels are increased in the cerebrospinal fluid of stroke patients. Hughes et al. (2002)[63] showed that mice deficient in MCP-1 develop less infarct volume as a consequence of focal brain ischemia . Dimitrijevic et al. (2007)[64]confirmed that mice deficient in the gene for the MCP-1 receptor, CCR2 transient focal cerebral ischemia results in reduced infarct size, edema, leukocyte infiltration and expression of inflammatory mediators.The results in the present study are in agreement with those reported byHughes et al. (2002) ; Soriano et al. (2002) ; Kumai et al. (2004)63,65,66 who found that the level of a variety of chemokines increase in animal models of cerebral ischemia and their inhibition or deficiency has been associated with reduced injury.

Effect of Global Cerebral I/R on Intercellular adhesion molecule-1 (ICAM-1):

In the present study a significant increase in Intercellular adhesion molecule-1 (ICAM-1) level (P < 0.05) was found in the I/R rats as compared with sham group. The results in the present study are in agreement with those reported by Staunton et al. (1988)[67] who found that transient cerebral ischemia induces expression of ICAM-1 as a homodimer on the membrane of inflamed cerebral endothelial cells. And Soriano et al. (1996)[68] showed that neutrophil adhesion in ischemic areas may be deleterious and that ICAM-1 deficiency reduces neurological damage after transient focal cerebral ischemia,alsoConnolly et al. (1996) & Kitagawa et al. (1998)[69,70] showed that ICAM-1-deficient mice have smaller infarcts compared to wild-type mice following focal cerebral ischemia .

Effect of Global Cerebral Ischemia Reperfusion Injury on Brain Histopathology:

There was statistically significant difference between control group and normal sham group (P< 0.05) . The score of the control group shows sever cerebral injury and moderate injury. Shah et al. (2005)[51] showed that in MCA/BCA occlusion for (30 min.) and then following reperfusion for (1 hour.), caused marked congestion of blood vessels.These effects were further augmented following reperfusion i.e.lymphocytic proliferation and neuronal necrosis.Chandrashekhar et al. (2010)[54]confirmed that the global cerebral ischemia on Sprague–Dawley rats by bilateral carotid artery (BCA) occlusion for 30 min followed by 1 hour reperfusion caused marked congestion of blood vessels and neutrophil infiltration and neuronal necrosis.

Measurement of Cerebral infarction area after Global Cerebral Ischemia Reperfusion Injury by 2,3,5-triphenyltetrazolium chloride (TTC) staining:

In the present study, measurement of Cerebral infarction area after Global Cerebral Ischemia for 30min. and Reperfusion 1hour using 2,3,5 triphenyltetrazolium chloride (TTC) staining.That showed a significant increase in cerebral infarction area of control group as compared to treated groups.These results are in line with Chandrashekhar et al. (2010) ; Prakash et al. (2011) and Lapi et al. (2012)[54,71,72]

The Effect of treatment on Study Parameters Effect of Amlodipine on Inflammatory Markers (IL-6, IL-9,MCP-1 and ICAM-1):

We found that pretreatment with amlodipine for (10) days before cerebral ischemia result in significant (p<0.05) decrease in inflammatory mediator,such as proinflammatory cytokine IL-9 , IL-6, chemokine MCP-1 and intercellular adhesion molecule-1,ICAM-1.Yoshii et al. (2006)[36] showed regression of atherosclerosis, in apolipoprotein E–deficient(ApoEKO) mice by amlodipine through inhibitory actions on oxidative stress, inflammation and the production of adhesive molecules ,and found mice that were treated with amlodipine suppressed in MCP-1 ,and ICAM-1.Martinez-Martin et al. (2011)**73**studied the effects of treatment with olmesartan/amlodipine and olmesartan/hydrochlorothiazide on inflammatory and metabolic parameters (including new-onset diabetes as a secondary endpoint) **.** Both olmesartan-based combinations were effective, but the amlodipine combination resulted in metabolic parameters and anti-inflammatory effects that may have advantages over the hydrochlorothiazide combination **.** In this study the treatment with olmesartan/amlodipine showed a significantly reduction of the inflammation markers such as IL-6 ,ICAM-1**.**To

the best of our knowledge, this study is the first to have measured IL-9 in rat model of cerebral ischemia reperfusion injury**.**

Effect of Amlodipine on Brain Histopathology:

In the present study the pretreatment with amlodipine for (10) days before cerebral ischemia caused improves the brain injury significantly (P< 0.05) as compared with control (induced untreated) group. The score of the control group shows sever cerebral injury while the score of amlodipine treated group shows normal and mild injury . That confirmed results reached by Halici et al. (2008)[74]who evaluated the effects of amlodipine as an antioxidant and analyze the histopathologic changes in experimental ischemic and ischemic-reperfusion (I/R) injury in rat ovaries,and their results showed that conservative treatment with amlodipine is effective in reducing tissue damage induced by ischemia, ischemic-reperfusion (I/R), or both in ovaries. Mogi et al. (2006)**39** found that amlodipine treatment reduces stroke size and neurologic deficit after focal brain ischemia, possibly through an increase in cerebral blood flow and inhibition of superoxide production.

Effect of Amlodipine on Cerebral infarction areaby 2,3,5-triphenyltetrazolium chloride (TTC) staining:

We found that the pretreatment with amlodipine for (10) days before cerebral ischemia result in significant (P<0.05) reduction infarct sizefrom global cerebral ischemia/reperfusion as compare with control(induced- untreated) group.These findings are in line with Mogi et al. (2006) [39] who found that amlodipine treatment reduces stroke size after focal brain ischemia that was measured by 2,3,5-triphenyltetrazolium chloride staining (TTC) staining**.**

The Effect of Candesartan on Study Parameters:

Effect of Candesartan on Inflammatory Markers (IL-6, IL-9,MCP-1 and ICAM-1):

In this study, pretreatment with candesartan for (10) days before cerebral ischemia result in significant (p<0.05) decrease in inflammatory mediator, such as proinflammatory cytokine IL-9 , IL-6 chemokine MCP-1 and intercellular adhesion molecule-1,ICAM-1.Results of the present study indicated that AT1 receptor blocked with candesartan can decrease ischemic brain injury . These findings are in line with Nishimura et al. (2000)[75] demonstrated an important role for Ang II in cerebrovascular control and indicate that therapeutic inhibition of the central Ang II system, and in particular pretreatment with selective AT1 receptor antagonists such as candesartan, could reduce neuronal injury resulting from cerebral ischemia.Ito et al. (2001a)76indicated that pre-treatment with AT1-antagonists such as candesartan could be of benefit in the prevention and treatment of brain ischaemia.AT1 receptor blockade and upregulation of eNOS activity can decrease ICAM-1 expression [77,78].Ando et al. (2004)[45]showed that antiinflammatory effects of AT1 receptor antagonists may be an important mechanism in protecting against ischemia.AlsoSchulz et al. (2006)[79] showed that AT1-receptor blockade reduces cerebral ischaemia-reperfusion injury in part by attenuating inflammatory processes .Tummala et al. (1999) &Brasier et al. (2002)[80,81]found that in kidney and other organs, AngII stimulates expression of proinflammatory mediators, including growth factors, cytokines and chemokines, and adhesion molecules.There are few published reports concerning the inflammatory marker (IL-9) in cerebral ischemia reperfusion injury.To the best of our knowledge, this study is the first to have measured IL-9 in rat model of cerebral ischemia reperfusion injury.

Effect of Candesartan on Brain Histopathology:

In the present study, pretreatment with candesartan for (10) days before cerebral ischemia ameliorated the brain injury significantly (P < 0.05) as compared with control group. The score of the control group showed sever cerebral injury while the score of candesartan treated group showed normal and mild injury .Ozacmak et al. (2007)[82]showed that treatment with candesartan caused remarkable reduction in these degenerative changes on CA1 neurons in hippocampal region after bilateral occlusion of CCAs.

Effect of Candesartan on Cerebral infarction areaby 2,3,5-triphenyltetrazolium chloride (TTC) staining:

In the present study, pretreatment with candesartan for (10) days before cerebral ischemia result in significant (P<0.05) reduction infarct size, compared with control group,and Cerebral infarction area was

measured by using 2,3,5 triphenyltetrazolium chloride (TTC) staining.Nishimura et al. (2000) [75] showed that pretreatment with the AT1-receptor blocker candesartan resulted significantly reduced infarct size after transient cerebralischemia in hypertensive and normotensive rats , and Cerebral infarction area was measured by using 2,3,5triphenyltetrazolium chloride (TTC) staining **.**

The Effect of Combination of amlodipine and candesartan on Study Parameters 4.2.3.1 Effect of Combination on Inflammatory Markers (IL-6, IL-9,MCP-1 and ICAM-1):

In the present study, pretreatment with Combination of candesartan and amlodipine for (10) days before cerebral ischemia result in significant ($p<0.001$) decrease in inflammatory mediator,such as proinflammatory cytokine IL-9 , IL-6, chemokine MCP-1 and intercellular adhesion molecule-1,ICAM-1.We found, in this study, that Pre-treatment with combination of candesartan & amlodipine more effective than monotherapy treated of candesartan or amlodipine alone.

Toba et al. (2006)83 showed that Ang II has been shown to enhance the expression of ICAM-1, and MCP1 in vascular endothelial cells . Harrison et al. (2003) [84], reported that pretreatment with amlodipine decreased the overexpression of ICAM-1, and MCP-1.

Takai et al. (2011)85stated that candesartan and amlodipine combination therapy could have a powerful protective effect in vascular tissues via the reduction of oxidative stress.

Dong et al. (2011)86showed that benefit effect of a combination of valsartan , and amlodipine were associated with an additive improvement in CBF, and improvement in cerebral arteriolar remodeling and vascular endothelial dysfunction.

Erdine (2012)87demonstrated that olmesartan plus amlodipine has effects beyond BP lowering by showing beneficial effects on markers of inflammation, endothelial dysfunction and oxidative .

Zhou et al. (2004)88showed that amlodipine has antihypertensive and antioxidant activity in vivo, which effectively inhibits many of the oxidative stress-dependent mechanisms involved in Ang II-mediated cardiovascular injury.

Effect of Combination of amlodipine and candesartan on Brain Histopathology:

We found that pretreatment with combination candesartan& amlodipine for (10) days before cerebral ischemia ameliorated the brain injury significantly ($P < 0.001$) as compared with control (induced - untreated) group. The score of the control group shows severe cerebral injury while the score of combination candesartan &amlodipine treated group shows normal score and mild injury. To the best of our knowledge,there is no previous study to investigate the effect of combination candesartan & amlodipine on Brain Histopathology of global cerebral ischemia – reperfusion injury in animal model .

Effect of Combination of amlodipine and candesartan on Cerebral infarction area by 2,3,5-triphenyltetrazolium chloride (TTC) staining:

In the present study, the Cerebral infarction area was measured by using 2,3,5triphenyltetrazolium chloride (TTC) staining,and result in significant ($P<0.001$) reduction infarct size compared with control(induced-untreated) group . To the best of our knowledge, this study is the first measured infarct size by TTC stain after treatment by combination candesartan& amlodipine .

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