



Anti-oxidant, Anti-inflammatory and Anti-apoptotic Effect of Adiponectin on Cerebral Ischemia Reperfusion Injury in Rats

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Received: 10 January 2019; | Revised: 30 January 2019; | Accepted: 10 June 2019

Abstract

Background: Ischemic stroke is a major cause of death and disability worldwide. Despite decades of intense research, the beneficial treatment of stroke remains limited. Adiponectin, a circulating adipose-derived hormone, has beneficial actions on cardio- and cerebrovascular disorders. A low level of plasma adiponectin is associated with ischemic cerebrovascular disease. In the present study, the role of adiponectin in the pathogenesis of acute cerebral injury was investigated. Rats were divided into three groups: (i) Sham operated group; (ii) Ischemia/reperfusion (I/R) group, rats were subjected to one hour middle cerebral artery occlusion followed by 23 hours of reperfusion (I/R); (iii) Adiponectin-treated group, adiponectin (0.5 mg/kg) was injected through jugular vein 10 minutes after the onset of reperfusion. Adiponectin supplementation improved neurological function and resulted in reduction in infarction size, and brain edema, brain contents of malondialdehyde (MDA), brain levels of nitric oxide (NO) levels, tumor necrosis factor-alpha (TNF- α) and caspase-3. Moreover it enhanced SOD (superoxide dismutase). Results of the present study demonstrate that adiponectin exerts a potent cerebroprotective effect against I/R injury through its anti-inflammatory and anti-oxidant action and anti-apoptotic mechanisms. It might be a potential target for ischemic stroke therapy.

Keywords: Ischemic stroke, Adiponectin, Cerebroprotective

List of abbreviations

BBB; Blood brain barrier; CNS, Central nervous system; ELISA, Enzyme linked immunoassay; eNOS, Endothelial nitric oxide synthase; iNOS, Inducible nitric oxide synthase, I/R, Ischemia/Reperfusion; MCA, Middle cerebral artery;

MDA, Malondialdehyde; nNOS, Neuronal nitric oxide synthase; NO, Nitric oxide; SOD, Superoxide dismutase; TNF- α , Tumor necrosis factor alpha; tPA, tissue plasminogen activator; ROS, Reactive oxygen species.

1. Introduction

Cerebral ischemic injury is a major cause of death and disability all over the world [1]. It is the third leading cause of death, after cardiovascular diseases and cancer [2]. Ischaemia-reperfusion injury (I/R) of the central nervous system (CNS) is a complex interplay between biochemical, vascular and cellular factors [3] that may occur after cerebral ischemia, traumatic head injury, carotid endarterectomy or aneurysm repair [4].

Cerebral ischemia is a major type of stroke, accounting for about 80% of stroke cases [1]. Ischemic stroke results from a temporary or permanent reduction of cerebral blood flow that leads to structural and functional damage in different brain regions during ischemia [5] and reperfusion [6]. Restoration of blood supply, referred to as reperfusion is a desired goal for acute stroke treatment. Reperfusion can be achieved either by using thrombolytic reagents such as tissue plasminogen activator (tPA), or through mechanical removal of thrombi. Spontaneous reperfusion also occurs after ischemic stroke. However, despite the beneficial effect of restoring oxygen supply by reperfusion, it also causes deleterious effect compared with permanent ischemia. The main mechanisms of reperfusion injury include oxidative stress, mitochondrial mechanisms, leukocyte infiltration, platelet activation and aggregation, inflammation, complement activation, apoptosis and blood-brain-barrier (BBB) disruption, which ultimately lead to brain edema or hemorrhagic transformation and eventually causing significant neuron death and neurological dysfunctions [7, 8].

Despite decades of intensive research, the beneficial treatment of stroke remains limited [9]. In light of this, the search for effective means ameliorating cerebral ischemia-reperfusion injury is one of the major problems of experimental medicine and biology [10]. Emerging evidence suggests that adiponectin may be one such potential therapy [11, 12].

Adiponectin is one of adipose cell-derived protein hormones secreted by the adipose tissue, abundant in human plasma, and can be expressed in the bone marrow, cardiomyocytes, osteoblasts, and CNS [13]. It has anti-inflammatory, anti-oxidant, anti-atherogenic and antidiabetic effects, thus

exhibiting protective effects on cells, tissues and organs. It exerts its physiological functions by activating two membrane receptors, adiponectin receptor 1 and 2 that are prominently expressed in skeletal muscle, adipose tissue, liver, and brain [14, 15].

Recently, a cross-sectional study showed that plasma adiponectin levels are decreased in patients with ischemic cerebrovascular disease [16]. Furthermore, a clinical study has suggested an association between hypoadiponectinemia and increased mortality after ischemic stroke and a negative correlation between adiponectin levels and initial infarct volume. However, the involvement of adiponectin in cerebrovascular disease and its potential mechanisms have not been completely elucidated [17].

So we aimed in this study to investigate the neuroprotective role of adiponectin against the development of ischemic stroke in the rat model of focal cerebral I/R injury and its potential underlying mechanisms focusing on its possible anti-oxidative, anti-inflammatory and anti-apoptotic effects.

2. Materials and Methods

2.1 Animals

Thirty adult male albino Wistar rats weighing 230 - 250 g were housed in plastic cages at a controlled temperature (25 ± 3 ° C), (50 ± 10 %) humidity, with alternating 12 h light and dark cycles. They were allowed to standard chow pellets and drinking water ad libitum. The animals were left to accommodate for one week before the experiment. Experiments were performed between 8 and 10 a.m. This work was carried out according to the international guidelines for care and use of laboratory animals, and the experimental protocol was ethically approved by the Animal Care Committee, Faculty of Medicine, Benha University.

2.2 Experimental design

Thirty Rats were randomly divided into three groups (n = 10 per group):

(i) Sham operation group, rats were subjected to the surgical procedures without ischemia reperfusion (I/R).

(ii) Ischemia/reperfusion (I/R) group, rats were subjected to 1 h middle cerebral artery occlusion followed by 23 h reperfusion (I/R).

(iii) Adiponectin-treated group (I/R + Adiponectin), adiponectin (0.5 mg/kg) was administered through jugular vein 10 minutes after the onset of reperfusion. Adiponectin was provided by Sigma Aldrich, (St. Louis, MO, USA).

2.3 Induction of middle cerebral artery occlusion

The rat model of left cerebral ischemia-reperfusion was established according to a previously published method of Longa et al. (1989). Briefly, overnight-fasted rats were anesthetized with 10% chloral hydrate (3.5 ml/100 g, i.p. injection; Tianjin Kemiou Chemical Reagent Co., Ltd., Tianjin, China). After the skin was exposed through a midline neck incision, the left common carotid artery, external carotid artery, and internal carotid artery were exposed and isolated through a ventral midline incision. After electrocauterization of the occipital artery, the external carotid artery, distal to the heart, was ligated with two surgical sutures. The common carotid artery and internal carotid artery were clamped. The silk sutures around the external carotid artery were lifted toward the common carotid artery ensuring that the external carotid artery and common carotid artery were placed at the same plane. A gap was then made at the bifurcation of the external carotid artery adjacent to the common carotid artery, and a monofilament suture (Beijing Sunbio Biotech Co., Ltd., Beijing, China) was slowly inserted from the incision close to the internal carotid artery. The suture was then withdrawn from the internal carotid artery until resistance was felt at the bifurcation of the external carotid artery. Furthermore, the suture tip almost reached the left middle cerebral artery, thereby occluding blood flow it. The suture was fixed and the wounds were then sutured. The animals were subsequently returned to their cages (at 26 ° C). After one hour of ischemia, rats were re-anesthetized via ether inhalation. The suture was then lifted slightly until the resistance was felt indicating that the suture tip had reached the external carotid artery. Blood flow in the left middle cerebral artery resumed as a result. Reperfusion was allowed for 23 hours. Rectal temperature was maintained between 36.5 ° C and 37.2 ° C with a

homeothermic blanket to avoid cerebral hypothermia. Successful establishment of the model was confirmed by the right forelimb flexion or right side circling after the animals recovered from the anesthesia. The same protocol was applied for sham-operated rats, but only the arteries were isolated [18].

2.4 Mortality score and neurological deficit score

The mortality of rats in each group was calculated according to the formula: Percentage mortality = number of deaths in the group/total number of rats in the group × 100%. After one-hour ischemia and 23-hour reperfusion, neurological deficits of rats were evaluated according to a previously described 5-grade scale [18]. The scale was graded as follows: 0 point: rats have no neurological symptoms, 1 point: minor defects (rats cannot fully extend the contralateral forepaw), 2 points: moderate defects (rats circle toward the contralateral side), 3 points: severe defects (rats fall toward the contralateral side), 4 points: rats cannot walk spontaneously or depressed level of consciousness.

2.5 Measurement of brain water content

Cerebral edema was determined by measuring the brain water content according to the wet - dry method [19]. After 23 h of reperfusion, animals were anesthetized with 10% chloral hydrate (3.5 ml/kg) and decapitated. The brains were rinsed with saline and separated into ischemic and non-ischemic hemispheres, then immediately weighed to gain the wet weight (WW). The brains were placed in an oven at 100 ° C for 24 h and weighed to obtain the dry weight (DW). The brain water content (%) was measured using the following formula: $(WW - DW) / WW \times 100\%$.

2.6 Measurement of the infarction size

The brains were carefully dissected out on ice. Coronal brain sections (2 mm thickness) were made from the forebrain region and immersed with 2% 2, 3, 5 triphenyltetrazolium chloride solution at 37 ° C for 30 min. Infarct size was calculated according to the following formula:

Percentage infarct size = the volume of the cerebral hemisphere on the normal side - The non-

infarct area on the ischemic side/the volume of the cerebral hemisphere on the normal side $\times 100\%$. [20]

2.7 Tissue collection

Brain samples were washed with isotonic saline and homogenized in either, ice-cold saline solution for malondialdehyde (MDA) and nitric oxide (NO) determination, or phosphate buffered saline for other parameters. Supernatant was obtained by centrifuging the homogenate at 12000 rpm for 20 min [21].

2.8 Assessment of brain lipid peroxides content measured as malondialdehyde (MDA)

As a major indicator of oxidative stress, lipid peroxides were measured as MDA level in the brain homogenates. The principle of the assay depends on the reaction of MDA with thiobarbituric acid in acidic medium when incubated for 45 min at 95 ° C. The resultant pink color was extracted by n-butanol and the absorbance was measured at 535 nm by a spectrophotometer (UV-1601; Shimadzu, Kyoto, Japan) [22].

2.9 Assessment of brain nitric oxide (NO) content measured as total nitrates/nitrites

This assay determines total nitric oxide based on the chemical reduction of nitrate to nitrite by vanadium trichloride, followed by the colorimetric detection of nitrite as an azo dye product of Griess reaction at 540 nm [23].

2.10 Determination of brain superoxide dismutase (SOD) activity

SOD activity was determined by commercial kit (R&D Systems, Inc., USA), according to the method of Beauchamp and Fridovich (1971). The assay is based on the conversion of nitro blue tetrazolium (NBT) to NBT-diformazan by superoxide ions generated from the reaction of xanthine and oxygen catalyzed by xanthine oxidase. SOD reduces superoxide ion concentration, and hinders the appearance of NBT-diformazan. The absorbance was measured at 550 nm. SOD activity in samples was determined from a standard curve [24].

2.11 Assessment of brain tumor necrosis factor alpha (TNF- α) content

TNF- α content were determined in brain homogenates by rat specific RayBio TNF-alpha ELISA kits (RayBiotech, Inc., USA). The assay procedure was followed as mentioned in the manuals and the absorbance of the yellow color was measured at 450nm [25].

2.12 Assessment of brain caspase-3 level

The level of brain caspase-3 was measured by a USCEN ELISA kit (Life Science Inc., PRC). The finally developed yellow color was measured at 450 nm using a microplate reader [20, 21].

2.13 Statistical analysis

All analyses were performed using the program Statistical Package for Social Sciences version 19 (SPSS Inc, Chicago, IL, USA). The data are presented as the mean \pm standard deviation (SD). Comparisons between groups were analyzed by using one-way Analysis of Variance (ANOVA) followed by post-hoc LSD test. Probability of chance (P value) < 0.05 was considered statistically significant.

3. Results

3.1 Effect of adiponectin on the mortality rate and neurological function in rats exposed to cerebral I/R injury

The mortality percentage was increased from 0 in sham operated group to 30 % in I/R group and it decreased by adiponectin administration to be 10%. As shown in table 1, the neurological deficit scores were significantly increased from 0 in sham operated group to 2.83 ± 0.18 in I/R group ($P < 0.05$). Adiponectin administration significantly decreased the neurological deficit to 1.15 ± 0.06 ($P < 0.05$) as compared with I/R group.

3.2 Effect of adiponectin on brain water content in rats exposed to cerebral I/R injury

As shown in table 1 and fig 1, the brain water content in the ischemic area of I/R group were significantly increased from 54.50 ± 4.32 in sham operated group to 85.12 ± 6.96 in I/R group ($P < 0.05$). Treatment with adiponectin significantly

decreased the brain water content to 60.85 ± 5.13 ($P < 0.05$) when compared with I/R group.

3.3 Effect of adiponectin on cerebral infarction size in rats exposed to cerebral I/R injury

As shown in table 1 and fig. 1 & 2, cerebral I/R induced a significant increase in cerebral infarction size from 0 in the sham operated group to 70.01 ± 1.67 in I/R group. Administration of adiponectin induced a significant decrease in cerebral infarction size to 32.33 ± 0.64 compared to I/R group ($P < 0.05$).

3.4 Effect of adiponectin on total lipid peroxides measured as malondialdehyde (MDA) in rats exposed to cerebral I/R injury

As shown in table 2 and Fig. 3, cerebral I/R resulted in a significant increase in brain MDA content from 2.68 ± 0.22 in the sham operated group to 6.75 ± 0.55 ($P < 0.05$). Adiponectin induced a significant decrease in brain MDA contents to 3.01 ± 0.32 as compared to I/R group ($P < 0.05$).

3.5 Effect of adiponectin on brain nitric oxide (NO) content measured as total nitrate/nitrites in rats exposed to I/R injury

Table 2 and fig. 3 showed that brain NO content was significantly elevated following cerebral I/R from 9.62 ± 0.94 in sham operated group to 19.88 ± 1.23 ($P < 0.05$) and that administration of adiponectin caused a significant reduction in brain NO content to 11.45 ± 1.36 as compared to I/R group ($P < 0.05$).

Table 1 :Neurological function, infarction size, brain water content in all experimental groups

Groups	Group I (Sham operated)	Group II (I/R)	Group III (I/R + Adiponectin)
Parameter			
Neurological score	0	$2.83 \pm 0.18^*$	$1.15 \pm 0.06^\#$
Infarction size (%)	0	$70.01 \pm 1.67^*$	$32.33 \pm 0.64^\#$
Brain water content (%)	54.50 ± 4.32	$85.12 \pm 6.96^*$	$60.85 \pm 5.13^\#$

Data is expressed as mean \pm standard deviation, P . value = probability of chance, $P < 0.05$ is significant tested by using One-way analysis of variance (ANOVA) test and Post Hoc multiple comparisons (LSD test).

*Significant difference vs sham operated group

^\# Significant difference vs I/R group.

3.6 Effect of adiponectin on brain superoxide dismutase (SOD) activity in rats exposed to cerebral I/R injury

As shown in table 2 and fig. 3, a significant reduction in brain SOD activity was observed in cerebral I/R group from 2.45 ± 0.14 in the sham operated group to 0.99 ± 0.05 ($P < 0.05$). Administration of adiponectin to cerebral I/R exposed rats caused a significant enhancement of brain SOD activity to reach 1.96 ± 0.03 when compared to I/R group ($P < 0.05$).

3.7 Effect of adiponectin on brain tumor necrosis factor alpha (TNF- α) level in rats exposed to I/R injury

Table 3 and fig. 4 showed that brain TNF- α was significantly elevated in rats exposed to I/R as compared to the sham operated group from 49.76 ± 2.58 to 135.36 ± 3.87 ($P < 0.05$). Treatment of rats, exposed to I/R, with adiponectin caused a significantly reduction in brain TNF- α level to 63.34 ± 2.64 as compared to I/R group ($P < 0.05$).

3.8 Effect of adiponectin on brain caspase-3 level in rats exposed to cerebral I/R injury

As shown in table 3 and fig. 4, cerebral I/R caused a significant enhancement in brain caspase-3 level as compared to sham operated group from 10.18 ± 0.77 to 28.22 ± 0.46 . Adiponectin administration to cerebral I/R exposed rats showed a significant decrease in brain caspase-3 level to 12.06 ± 0.38 as compared to I/R group ($P < 0.05$).

Table 2 :Brain content of MDA, NO and SOD in all experimental groups

Groups	Group I (Sham operated)	Group II (I/R)	Group III (I/R + Adiponectin)
Parameter			
MDA (mM/100 mg tissue)	2.68 ± 0.22	6.75 ± 0.55 *	3.01 ± 0.32 #
NO (mM/100 mg tissue)	9.62 ± 0.94	19.88 ± 1.23 *	11.45 ± 1.36 #
SOD (U/100 mg tissue)	2.45 ± 0.14	0.99 ± 0.05 *	1.96 ± 0.03 #

Data is expressed as mean ± standard deviation, *P*. value = probability of chance, *P*< 0.05 is significant tested by using One-way analysis of variance (ANOVA) test and Post Hoc multiple comparisons (LSD test).

*Significant difference vs sham operated group

Significant difference vs I/R group.

Table 3 :Brain levels of TNF-α and caspase 3 in all experimental groups

Groups	Group I (Sham operated)	Group II (I/R)	Group III (I/R + Adiponectin)
Parameter			
TNF-α (Pg/100 mg tissue)	49.76 ± 2.58	135.36 ± 3.87 *	63.34 ± 2.64 #
Caspase-3 (ng/100 mg tissue)	10.18 ± 0.77	28.22 ± 0.46 *	12.06 ± 0.38 #

Data is expressed as mean ± standard deviation, *P*. value = probability of chance, *P*< 0.05 is significant tested by using One-way analysis of variance (ANOVA) test and Post Hoc multiple comparisons (LSD test).

*Significant difference vs sham operated group

Significant difference vs I/R group.

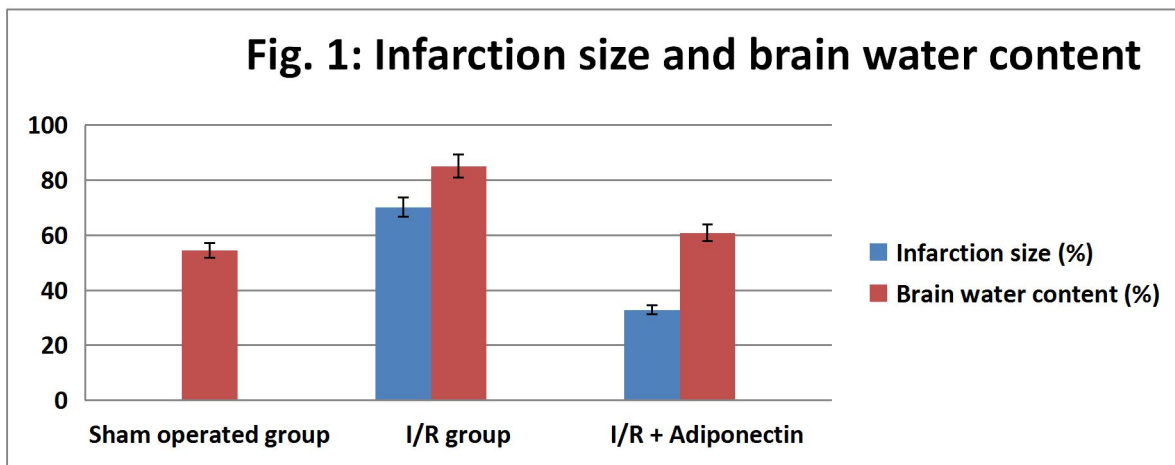


Figure 1 : Infarction size and brain water content

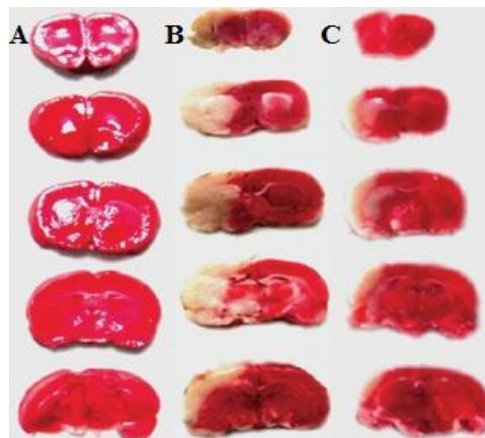


Figure 2 : Infarction size in all experimental groups (A) Sham operated group, (B): I/R group and (C): I/R + Adiponectin

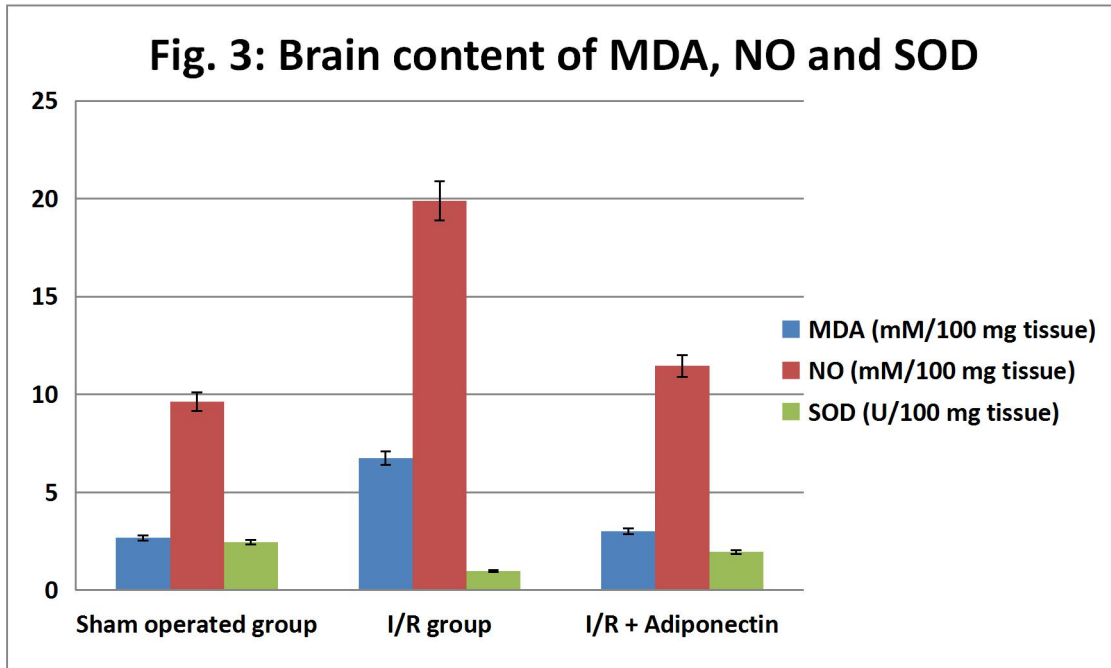


Figure 3 : Brain content of MDA, NO and SOD

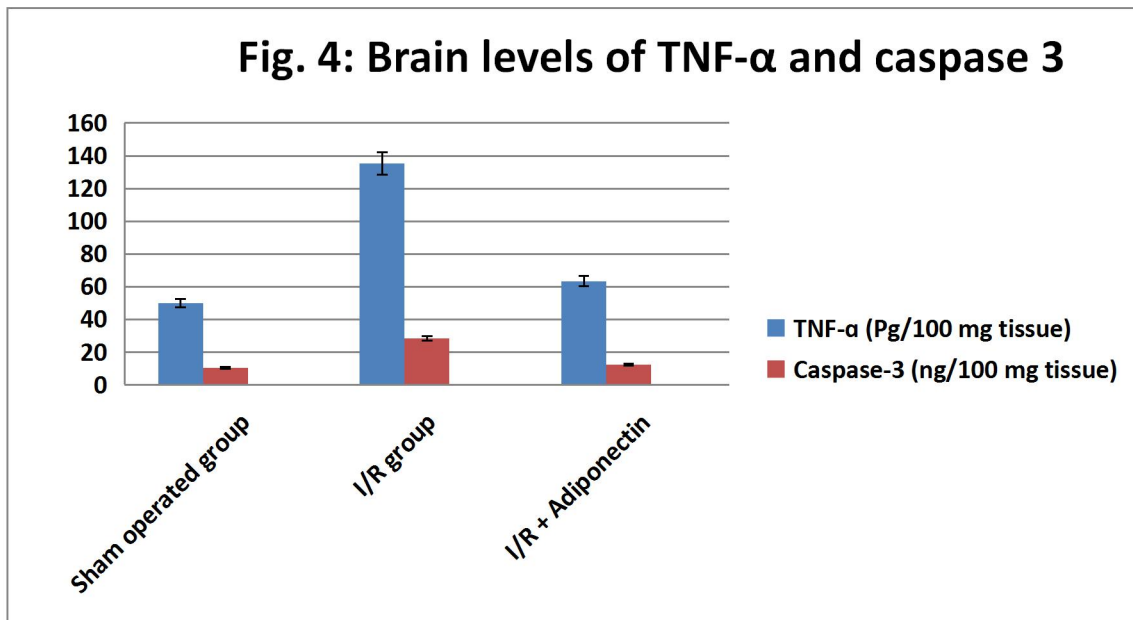


Figure 4 : Brain levels of TNF-α and caspase 3

4. Discussion

Cerebral ischemic injury is the most common cerebrovascular disorder. The association between adiponectin and cerebral ischemia remains unclear. Epidemiological studies have shown that hypoadiponectinemia could be a useful biomarker for the presence of ischemic stroke and increased mortality after ischemic stroke [16, 17]. Plasma adiponectin values may help predict neurological

severity and functional outcome in ischemic stroke patients [26].

Thus in the present study, we demonstrate that an adipose-derived hormone, adiponectin, protects the brain from acute ischemic injury in a rat model of focal cerebral I/R done through MCA occlusion.

The current study showed a significant decrease of neurological function, induction of cerebral infarction and brain edema after one hour of ischemia and 23 hours of reperfusion. These

findings are consistent with those of Melani et al. (2006) [27]. In this research, we demonstrated that treatment with adiponectin could reduce brain infarct size and brain edema while improving neurological function after focal cerebral I/R injury. These findings were in agreement to the previous studies [11, 12, 28].

Oxidative stress, stemming from distortion of the equilibrium between production and scavenging of free radicals, is one of the most important pathological mechanisms underlying I/R injury. Cerebral I/R injury is characterized by overproduction of reactive oxygen species and exhaustion of brain natural defense systems with consequent neuronal deterioration and delayed neuronal death [21, 29]. Sudden bursts of free radicals and reactive oxygen species (ROS) observed during I/R injury accumulate and attack proteins, lipids and nucleic acids leading to lipid peroxidation, cellular membrane damage, impairment of cellular functions and DNA fragmentation [30, 31]. MDA is considered as the most sensitive marker of lipid peroxidation. The high brain lipid peroxides content and low concentration of endogenous antioxidants renders it highly susceptible to oxidative damage [32]. Beside oxidative stress, nitrosative stress is another pathological mechanism significantly contributing to neuronal death induced by excessive nitric oxide (NO) production through nitric oxide synthases (NOSs) [33]. The role of NO in pathogenesis of ischemic brain damage is controversial. Nitric oxide is considered as a double-edged sword. Some evidence has been accumulated that NO plays either neurotoxic or neuroprotective roles during the process of cerebral ischemia and reperfusion. The amount and the source of NO are crucial factors determining its effects in cerebral ischemia. Nitric oxide produced by endothelial nitric oxide synthase (eNOS) has been suggested to exert beneficial effects during cerebral ischemia-reperfusion [34]. Neuronal NOS (nNOS) and especially inducible NOS (iNOS) activities have been proposed to be damaging to the ischemic brain [35]. Cerebral ischemia up regulates inducible nitric oxide synthase (iNOS), producing excessive nitric oxide which reacts with superoxide to form peroxynitrite resulting in cellular machinery dysfunction and neuronal death [30, 36, 37]. The current study revealed a significant increase in MDA and NO and a

significant decrease in brain SOD level indicating the involvement of oxidative stress in MCA occlusion induced cerebral I/R injury. The antioxidant effect of adiponectin against I/R-induced brain damage can be attributed to its ability to reduce the elevated brain levels of MDA and NO together with enhancement of SOD in rats exposed to cerebral I/R. The finding of this study was in agreement to Nishimura et al. (2008) who provided a causal evidence that adiponectin exerts its cerebroprotective effect via an eNOS-dependent mechanism [12]. Song et al. (2013) demonstrated that the neuroprotective action of adiponectin may result from the promotion of antioxidant capacity by inhibiting the NADPH oxidase 2 signaling system [28].

Similarly, expression of inflammatory cytokines in brain tissues was detected after brain I/R injury [21, 38]. Oxidative stress activates the expression of pro-inflammatory cytokines [39]. One of the most important cytokines released during ischemia/reperfusion is TNF- α . This pro-inflammatory cytokine can contribute to the pathogenesis of I/R injury by induction of cell adhesion molecules thereby enhancing reactive oxygen species production, facilitating leukocyte infiltration, and exaggerating the inflammatory response with subsequent cerebral damage [40]. Blockade of TNF- α receptors could reduce brain infarct volume after transient focal ischemia in rats [41]. The current study also revealed a significant increase in brain TNF- α levels in MCA occlusion induced I/R injury. Adiponectin administration caused a significant reduction in the brain levels of TNF- α in rats exposed to cerebral I/R. Chen et al. (2009) demonstrated that adiponectin displays a potent cerebroprotective activity via an anti-inflammatory effect and that nuclear factor kappaB (NF- κ B) is a key component in this process [11]. Adiponectin suppresses systemic inflammation through upregulation of anti-inflammatory cytokines (e.g., interleukin (IL)-10 and IL-1 receptor antagonist) and suppression of proinflammatory cytokines (e.g. TNF- α and interferon- γ) [42]. Adiponectin also inhibits the interaction between the endothelium and leukocytes in cerebral I/R. Therefore adiponectin might prevent the secondary insult caused by the inflammatory reaction [43].

Activation of apoptotic mechanisms and controlled neuronal death were observed following cerebral I/R [44]. Apoptosis is achieved by a cascade of intracellular cysteine aspartase specific proteases, named caspases, that mediate cell death and inflammation. There are two types of caspases: initiator caspases (caspases 8, 10, 9, and 2) and effector caspases (caspases 3, 7 and 6). The effector caspases are activated by initiator ones [45]. Among all members of the caspase family, caspase-3 is most abundant in the brain [46]. Activation of caspase-3 is considerably implicated in ischemic cell death [47]. Caspase-3 has been found to increase soon after forebrain ischemia with a persistent increase after reperfusion [48]. Activation of caspase-3 results in cleavage of actin and proteins that are vital for DNA repair and cell stability, causing cell death primarily by apoptosis [49]. ROS binding of TNF- α take part in the induction of apoptosis and promotion of caspases activation leading to nuclear damage [50, 51]. In this study, MCA occlusion induced I/R injury caused a significant increase in caspase 3 level. Treatment of rats exposed to cerebral I/R with adiponectin led to a significant decrease in the brain levels of caspase-3 and this is congruent with the previous studies which found the role of adiponectin in attenuating endothelial and neuronal apoptosis [17, 36].

5. Conclusion

The present observations indicate that adiponectin acts as an endogenous modulator of cerebral I/R injury and this may be attributed to lower the levels of lipid peroxidation, anti-inflammatory cytokines and pro-apoptotic caspase-3 and enhancement of antioxidant enzymes. Thus, adiponectin could represent a potential therapeutic target for the prevention and treatment of ischemic stroke.

Conflict of interest

The authors declare that there is no conflict of interest.

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