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Effects of Resatorvid on brain ischemic reperfusion injury in rats

Muntadher Hameed Thwaini[1], Ahmed R[2], Rihab H. Al-Mudhafar[3], Najah R. Hadi[4]

[1]*College of Pharmacy, University of Karbalaa, Karbalaa, Iraq*

[2]*College of medicine, university of ALNaharin, Baghdad, Iraq*

[3]*Middle Euphrate cancer Research, Faculty of Medicine, University of Kufa, Iraq*

[4]*Professor, Department of Pharmacology & therapeutics, Faculty of Medicine, University of* Kufa, Iraq,

drnajahhadi@yahoo.com

Abstract

Background: Ischemic stroke is one of the major causes of mortality and disability worldwide. Limitation of cerebral blood flow due to thrombosis can cause ischemic stroke that in turn affect cellular homeostasis due to insufficient oxygen and nutrient supply. Aims: Re-establishment of cerebral blood flow can cause further deterioration of ischemic brain tissue by a series of inflammatory, apoptotic and oxidative events resulting in cerebral ischemic-reperfusion injury which in turn leads to neuronal cell death and neurological impairments. Methods and materials: This study was carried out to evaluate the potential neuroprotective effects of Resatorvid in a global model of cerebral ischemic reperfusion injury in rats. A 24 Wistar-albino rats (weighing 200-300 grams) were divided randomly into 3 groups (n= 8 in each group), a sham (negative control) group, control (ischemic reperfusion group) and Resatorvid group (1mg/kg) intraperitoneally. Rats were exposed to 30 minutes of ischemia followed by 1 hour of reperfusion. At the end of the reperfusion, rats were sacrificed and brain tissue samples were obtained for histopathological scoring and inflammatory markers measuring. Tissue levels of interleukin 1 beta (IL-1b), IL-6, IL-8, and tumor necrosis factor alpha (TNF-a) were significantly lower in Resatorvid pre-treated group as compared to control group ($p<0.05$) in addition the histopathological score in Resatorvid group were much lower than the control group. Results: we see that administration of Resatorvid can be useful preventive method in attenuating the degree of brain injury during ischemic reperfusion process as shown by a significant reduction of brain inflammatory markers and lower histopathological damage in comparison with control group. Conclusion: the results of the present study revealed that pre-treatment with Resatorvid could confer neuroprotection in global cerebral ischemic reperfusion injury due to it is anti-inflammatory and anti-apoptotic effects.

Keywords: Resatorvid, brain ischemic, ischemic reperfusion injury in rats.

1. Introduction

stroke are neurological pathologies that can lead to several disorders relating to the site, size and severity of the lesion, hence, can vary from one individual to another and produce many difficulties in the lives of patients with brain damage. According to the World Health Organization, cerebrovascular accidents (stroke) are the second leading cause of death and the

third leading cause of disabilities worldwide [1] Ischemic stroke accounts for about 70%–80% of all strokes, the most ischemic stroke is due to the middle cerebral artery occlusion (MCAO), resulting in the brain tissue damage in the affected region, which is followed by inflammatory and immune response [2] current therapeutic approaches, including antiplatelet and thrombolytic drugs, only partially ameliorate the clinical outcome of stroke patients because such drugs are aimed at preserving or restoring cerebral blood flow instead of preventing the actual mechanisms associated with neuronal cell death [3].hypoxic ischemic brain injury (HIBI) is associated with significant neurologic disability, ranging from mild cognitive deficits to minimally conscious and persistent vegetative states [4] in which a "two-hit" model, being determined by primary injury from immediate cessation of cerebral oxygen delivery (CDO2) and secondary injury occurring after resuscitation. Oxidative stress is thought to be the primary event during brain ischemic reperfusion(I/R) injury [5]because reperfusion stimulates an overproduction of reactive oxygen species (ROS), like hydrogen peroxide (H2O2), which cause oxidation of proteins, lipids, and DNA and can induce cell proliferation, growth arrest, apoptosis, and necrosis [6]Meanwhile, the dysfunction of superoxide dismutase (SOD) and glutathione peroxidase (which are a natural anti-oxidant) can compromise endogenous antioxidant defense mechanisms and further exacerbate oxidative stress and ischemic/reperfusion injury [7]. During an ischemic attack, local microglia cells, intrathecal macrophages and migrating macrophages will produce a massive amount of cytokines activation of macrophages and mast cells will leads to the release of different pro-inflammatory mediators such as tumor necrosis factor-alpha (TNF-a), interleukin-1beta (IL-1 b), and interleukin 6 (IL-6) [8]elevated production of IL-6 contributes to the pathogenesis of different autoimmune and inflammatory diseases like inflammatory bowel disease and others [9]cytokines are a small glycoproteins molecules that may act as pro-inflammatory mediators like interleukin 6(IL-6), interleukin 1(IL-1), tissue necrosis factor alpha (TNF) that will aggravate brain I/R injury [10].The pro-inflammatory cytokine cascade appears to involve in the beginning the release of IL-1 and TNF-a, that in turn leads to the later release of other pro-inflammatory cytokines such as IL-6 and IL-8, activation and infiltration of leukocytes, and the production of anti-inflammatory cytokines including IL-4 and IL-10, that will produce a negative feedback on the cascade [11]. IL-10 which is produced by microglia and macrophages has a protective role in brain I/R injury by promoting the survival of viable neurons and aiding in resolving inflammation [12]. So the Stroke-related cytokines are TNF-α, IL-1, IL-6, IL-20, IL-10, transforming growth factor-β (TGF-β), IL-8 and interferon inducible protein-10 (IP-10), Whereas IL-1β, IL-8, MCP-1, and TNF-α appear to exacerbate cerebral injury, the anti-inflammatory cytokines TGF-β, IL-10, and IL-1 receptor antagonist (IL-1Ra) appear to be neuroprotective [13]. Elevated production of IL-6 contributes to the pathogenesis of different autoimmune and inflammatory diseases, Regarding stroke, there is a significant relation between early intrathecal production of IL-6 and the subsequent size of the brain lesion which can be used as a prognostic tool, predicting the size of the brain damage before it is possible to accurately visualize it with radiological means [14]. IL-8 has many roles in brain injury including induction of lysosomal enzyme release from Neutrophils [15] expression of adhesion molecules on Neutrophils [16] and adherence of Neutrophils to unstipulated endothelial cells with chemotactic activity for T lymphocytes [17] while the elevation of IL-1b will lead to astrogliosis and apoptotic cell death in the brain of rats, additionally, IL-beta will also significantly reduce the number of developing oligodendrocytes and impair mylenation [18] TNFa can cause brain tissue edema, destruction of blood brain barrier (BBB) and disruption of endothelial barrier integrity [19]. Resatorvid or TAK242 was firstly discovered by Takeda

Pharmaceutical Company Limited (Osaka, Japan) and was studied in many clinical trials in many countries like Japan and European union as a potential new antisepsis drug by acting as a cytokine production inhibitor [20] It is a drug that acts as a selective antagonist of the receptor toll like receptor 4 (TLR4) [21] that has anti-inflammatory [22], neuroprotective [23] and anticancer activities [24].Toll like receptor 4 (TLR4) is a very important innate immunity receptors which recognizes pathogen associated molecular patterns (PAMPs) and endogenous ligands to mediate frontier defense in the central nervous system (CNS) [25] intracellular pathogens can be eliminated via TLR4 induced Autophagy pathway which is considered (Autophagy) as both innate and adaptive immunity thus keeping normal homeostasis during pathogen infection [26] stimulation of this receptor via PAMPs from different sources (e.g.: bacterial lipopolysaccharide, flagellin, viral RNA) will leads to a complex inflammatory cascade the eventually disrupt CNS homeostasis [27]. TLR4 can activate two parallel signaling pathways for the initiation of transcription factor activation which is responsible for the regulation of the expression of pro-inflammatory cytokine genes through two distinct adaptor protein (myeloid differentiation factor88, ^Myd88^and TIR domain containing adaptor inducing interferon ^TRIFs^). The Myd88 pathway will activates signal transduction molecules that include interleukin associated kinases (IRAKs), tumor necrosis factor receptor associated factor 6(TRAF6) eventually causing the activation of nuclear factor kB (NF-kB) with the subsequent production of pro-inflammatory cytokines [28]. Preclinical data have showed that TLR4 has an important role in neuronal death and must be considered as a new therapeutic intervention for brain injury [29]. TAK-242 will bind to the Cys747 part in the intracellular domain of the TLR4 in thus inhibiting protein functionality [21], and due to the TAK-242 small molecular size and high lipophilicity , it will cross blood brain barrier rapidly making it good choice to be studied for it is neuroprotective ability.

Aim of the study

The main objective this study is to assess the potential neuroprotective effect of Resatorvid in a global model of cerebral ischemic reperfusion injury after bilateral common carotid artery occlusion (BCCAO) in rats by measuring the cerebral levels of IL-1b, IL-6, IL-8 and TNF-a using ELIZA technique, also to study the potential role of toll like receptor 4 in mediating these effect.

2. Material and method

A total of 24 adult male Wistar albino rats weighing (200-400g) were purchased from college of pharmacy-ALKufa University, they were housed in the animal house at the same university, the temperature of the animal house was maintained at about 25 C, the humidity was maintained at the range of (60-65%), with alternating 12 hour light – 12 hour dark cycles .till the beginning of the experiment, rats could freely access water and chow diet. The rats were distributed randomly into three groups as follow: Group 1 (sham group): in this group, the surgical and the anesthetic process were done without the bilateral common carotid artery occlusion (BCCAO). Group 2 (control group): in this group, BCCAO was performed for 30 min., then reperfusion was allowed for 1 hour, Group 3 (treatment group): in this group, Resatorvid 1mg/kg dissolved in 5% dimethyl sulfoxide [30] injected intraperitoneally 1 hour before induction of ischemia. A global model of brain ischemic/reperfusion injury was persuade by BCCAO [31] animal temperature was maintained at about 37 C by the use of a light bulb, and the rats were anesthetized by ketamine at a dose of 100mg/kg with lidocaine at a dose of 10mg/kg intraperitoneally [32] After being placed on the back and fixed firmly in the supine position, a small incision was performed

in the middle of the neck by fine surgical tool And the carotid arteries which exist underneath the trachea were isolated from the vagal nerves bilaterally and occluded by mini vascular clamps to induce ischemia, after 30 minutes of occlusion the clamps were removed and reperfusion was allowed for 1 hour. After 1 hour of reperfusion , the rats were decapitated and the brains were isolated and washed with ice cold phosphate buffer solution (PBS), they were kept on ice and weighed then sectioned into two coronal slices, the first one was kept in 10% formalin for histopathological analysis and the other one was mixed in 1:10 (w/v) ratio of ice cold 0.1 M PBS (PH7.4) that contain 1x cocktail protease inhibitor, and 0.2% triton X-100 then homogenized by ultrasonic liquid processor, the homogenates were then centrifuged at 15000 g for 30 minutes at 4 c and the supernatants were withdrawn and stored at -80 c for measurement of other markers by ELIZA technique [33]. The histopathological analysis was carried out by a senior pathologist who is blinded to the study design and the allocation of each animal and the scoring of brain damage was set as follow [34] 0: normal, no morphological signs of damage, 1: slight, edema or eosinophilic or dark (pyknotic) neurons or dark shrunk cerebral purkenje cells, 2: moderate, at least two small hemorrhages, 3: severe, clearly infracted foci (local necrosis).

3. Statistical analysis

The statistical analysis for this study was performed by the means of SPSS software (statistical package for social sciences) version 24, means with standard deviation were considered as descriptive measurement while one-way ANOVA was considered to test significant differences between more than 2 groups, in which post Hoc. Tukey test was used for multiple comparisons, statistical significance in all the tests was considered when p value $\leq 0.05\%$ [35]

4. Results

In order to evaluate the neuroprotective effects of Resatorvid, a number of inflammatory parameters were examined (IL-1b, IL-6, IL-8, TNF-a) after induction of global cerebral ischemia with or without pre-treatment with the previous agent.

Effect of resatorvid on inflammatory parameters cerebral levels: The cerebral concentration of inflammatory parameters was significantly ($p>0.05$) elevated in control group at the end of the study in comparison with sham group (table one). For Resatorvid treatment group, inflammatory parameters cerebral concentration was significantly ($p<0.05$) lesser than control group (except for TNF-a in which TAK242 did decrease the TNF-a levels as compared to control group but this decrease did not reach to statistical significance) as shown by table two and figure one:

Table (1): comparison of inflammatory parameters cerebral concentration between control group and healthy group, (no. of animal in each group=8) by using t* test

Variables	Control group (mean ± SD)	Healthy group (mean ± SD)	95% C.I of the difference	P value
IL-1b	56.44±2.82	27.3±2.18	26.44-31.84	0.0001*
IL-6	96.30±5.35	51.3±4.96	39.47-50.53	0.0001*
IL-8	192±39.17	147.2±27.70	8.42-81.18	0.019*
TNF-a	180.03±33.7	130.7±15.21	21.29-77.36	0.002*

Table (2): comparison of inflammatory parameters cerebral concentration between control group and Resatorvid group, (no. of animal in each group=8) by using t* test

Variables	Control group (mean ± SD)	Resatorvid group (mean ± SD)	95% C.I of the difference	P value

IL-1b	56.44±2.82	38.94±3.62	14.02-20.97	0.0001*
IL-6	96.30±5.35	63.76±3.63	27.64-37.44	0.0001*
IL-8	192±39.17	162.41±24.06	5.27-64.44	0.09*
TNF-a	180.03±33.7	163.85±23.21	14.84-47.20	0.28*

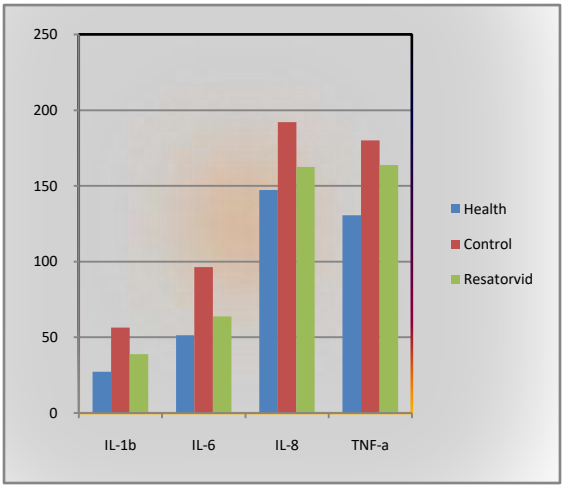

Figure (1): bar chart showing the difference in inflammatory parameters cerebral mean concentration among different groups

Histopathological findings: At the end of the study, rat brain sections of the three experimental groups were evaluated for acute cerebral injury. The results are as follows: **Sham group**: In this group, (100%) of rats presented normal brain morphology. **Control group**: the histopathological scores of (25%) of rats in this group showed moderate brain injury, while (75%) showed severe brain injury. **Resatorvid group**: 37% of rats in this group presented with normal brain histology while 62.5% presented with slight brain injury as shown in table (3).

Table (3): histopathological scores in different study groups

Histopathological Score	healthy		control		Resatorvid	
	n	%	n	%	n	%
normal	8	100%	0	0.0	3	37.5%
slight	0	0	0	0	5	62.5%
moderate	0	0	2	25%	0	0
sever	0	0	6	75%	0	0
total	8	100%	8	100%	8	100%

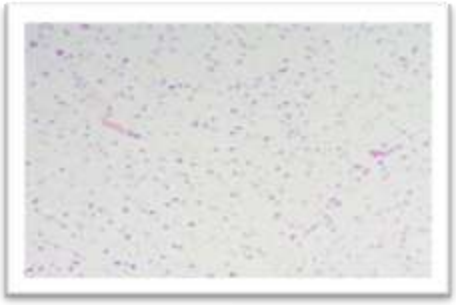

Figure (2): Photomicrograph of brain section of rat in the sham group, normal histology, score zero, H&E stain, 100x

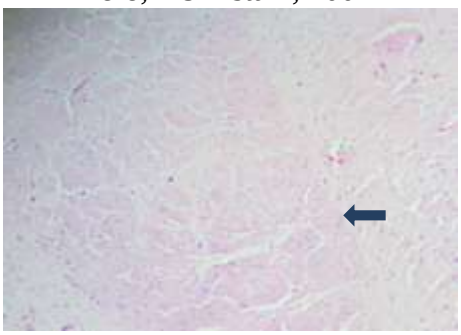

Figure (3): Photomicrograph of brain section of rat in the control group showing coagulative type necrosis (blue arrow) H & E, 100x

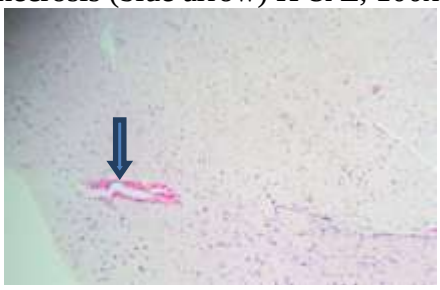

Figure (4): Photomicrograph of brain section of rat in the control group showing extravasations of RBC and congested vessel (blue arrow) H & E, 100x

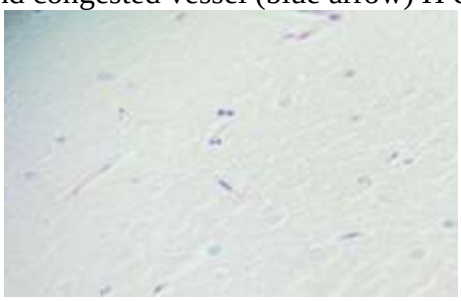

Figure (5): Photomicrograph of brain section of rat in the Resatorvid group showing edema and cellular swelling, H & E, 400x

5. Discussion

Resatorvid is extremely important to study about and develop in such subject due to the fact that brain I/R injury does not has a known efficacious management taking in consideration that all pharmacological interventions are aimed to target blood viscosity (either thinning the blood to remove a clot or a surgical procedure in case of haemorrhagic stroke to stop a bleeding)

without taking in consideration the quality of the reperfused blood coming back to the brain which will be full with cytokines (IL-1b, IL-6, IL-8, TNF-a etc), these cytokines could easily produce more damage even after the stroke ends and there cerebral levels can remain elevated for a long period , so it is extremely important not only to concentrate on restoring the blood flow but also to consider the possible effects of inflammatory cascade produced after, Resatorvid could be seen in the future as a solid elements in the guideline of managing stroke alongside the thrombolytic agents as more studies is made on it. .In this study, the pro-inflammatory cytokine TNF-a cerebral level was significantly increased in control group in comparison with sham group. TNF-a is a major pro-inflammatory cytokines which is released by macrophage, astrocytic, microglia and various neurons of brain tissue[36]TNF-a is produced in response to cerebral injury in a cascade reaction [37] TNF-a levels begin to increase after 30 minutes of occlusion maintaining such levels for up to 2 hours then normalized within 24 hour as has been shown in previous studies[38] which is consistent with this study. IL-6 is released in response to TNF-a, and secreted by T cells, astrocytic, microglia, and neurons [39] this study is in line with many studies including a 2005 study that established the elevation of IL-6 levels during brain damage in rats remaining high for 7 days [40] Inflammation has a key role in the propagation of ischemic cerebral injury as has been established by many studies revealing the deleterious effect of IL-1b in pathophysiology of stroke [41] Resident microglial cells considered to be the main source of IL-1b in early brain injury after an ischemic attack [42] The result of this study is in line with those reported by a 2010 study in which, it established a significant increase of IL-1b within first hour of reperfusion after Transient middle cerebral artery occlusion (tMCAO) in rats [43] regarding IL-8, it is considered to be a potent Neutrophil-activating chemotactic cytokine[44]In the present study, Resatorvid pre-treatment 1 hour before induction of transient global cerebral ischemia was found to decrease pro-inflammatory cytokines TNF-a, IL-6,IL-1b and IL-8 as compared to control group, but it did not decrease TNF-a enough to reach to statistical significance (p value was 0.28), it would do so probably if a larger sample were taken or if different conditions were established. Resatorvid ability to lower TNF-a levels also has been proven in amyotrophic lateral sclerosis mice where TAK242 has been given in a 2mg/kg dose ip [45]. Our observation in this study is consistent with a 2018 study that examined TAK242 ability to attenuates adverse neural effects of diet-induced obesity in mice with a 3mg/kg six days a week for 12 week ip, the final results has proved the above assumption by number of tests including TNF-a and IL-6 that were lower than the control group [46]. In another study, Resatorvid was used to evaluate it is hepatoprotection effects in rats that has been injured by Methotrexate in which, TAK242 was given in a dose of 5 mg/kg for 7 days followed by 7 days of oral MTX 0.2 mg/kg , the results stated that the TAK242 treated groups showed lower values of both inflammatory markers TNF-a and IL-6 than control group [47]. In a study that performed in Kufa university 2018 about the reno-protective effects of Resatorvid using two different doses (5 and 10 mg ip)/kg of rat body weight , the results of the study stated that TAK242 has the ability to lower inflammatory responses and IL-8 levels significantly after renal I/R injury as compared to control vehicle group [48]. TAK242 also was used in mice with their immune system manipulated to elicit some form of skin inflammation to evaluate TAK242 ability to antagonize such effects, the result of the study established the fact that TAK242 was able to lower inflammation and IL-1b efficiently as compared to control group [49]. Another study which was a cell line study that used a Human Microvascualr Retinal Endothelial Cells (HMVREC) and exposed it to increasing concentration of glucose to mimic the inflammatory process associated with diabetic nephropathy , a number of inflammatory cytokines were tested

and shown to be elevated (IL-1b, IL-8, TNF-a, monocyte chemoattractant protein- (MCP-) 1, vascular cell adhesion molecule- (VCAM-) 1, and intracellular adhesion molecule-(ICAM-) 1) but when TAK242 were implied along with the cell culture and glucose all the previous markers significantly dropped in level providing a solid evidence of TAK242 targeting such cytokines [50]. Regarding the histopathological examination, Resatorvid pre-treatment 1 hour before induction of transient global cerebral ischemia significantly ameliorated cerebral injury as compared to control group. The score of Resatorvid pre-treatment group showed normal brain histology and slight injury, while the control group showed moderate and sever injury. In a study where mice were used to evaluate the lung protection effects of TAK242 (intranasal route) in mice with combined allergic rhinitis and asthma syndrome (CARAS were induced using intranasal novalbumin) the histopathological findings stated that TAK242 significantly decreased eosinophil and monocytes lung infiltration leading to a much lower histopathological score as compared to control group [51]. TAK242 histopathological effects also had been proven in rats liver about study by decreasing infiltrated inflammatory cells and necrotic hepatocytes after 1 hour of liver ischemic reperfusion injury preceded with (30min) 1mg/kg of TAK242 [52]. Another study included the use of TAK242 in cardiac ischemic reperfusion injury in mice where TAK242 was administered in 3mg/kg dose at time of reperfusion, the histopathological findings stated that TAK242 treatment group manifested less cardiac remodeling and lower changes in the left ventricular dimensions (left ventricular end-diastolic diameter LVDD and left ventricular end-systolic diameter LVESD) as compared to control vehicle group [53].

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