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Histopathological Effects of L-Methionine in Rat Cerebral Ischemia Reperfusion I/R Injury

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ABSTRACT

Stroke is a serious condition in which specific area of the brain will loss the blood supply. When blood restored to the ischemic area the risk will be increased and brain cells will undergo cell death by the two known forms of cell death (apoptosis and necrosis)**.** Methionine is an essential amino acid needed by our body for many physiological pathways like synthesis of glutathione which is considered as more important endogenous antioxidant molecules. This study was designed to assess the possible neuroprotective activity of L-Methionine in cerebral I/R injury after bilateral common carotid artery occlusion (BCCAO) in rats. A total of 24 Adult Sprague-Dawley rats were used. They divided equally into four groups (sham, control, control vehicle and L-methionine treated groups). Their brains were removed and prepared by a reported procedure for histopathological study and staining with 2, 3, 5-triphenyltetrazolium chloride (TTC). It has been found that both histopathological and TTC staining results showed a significant role for L-Methionine as cerebroprotective agent and further studies are recommended to confirm that.

Keywords: Ischemia-Reperfusion, Stroke, Methionine, TTC.

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INTRODUCTION

Stroke is a serious condition in which specific area of the brain will loss the blood supply. when blood restored to the ischemic area the risk will increased[1] and brain cells will undergo cell death by the two known forms of cell death (apoptosis and necrosis) [2], and this usually occur in patient with high risk diseases like atherosclerosis or accompanied with stressful conditions. The prevalence of stroke increases with the increasing in the severity of life. Stroke considered as very critical state because it usually associated with long standing disabilities, inflammatory response and production of free radicals are usually associated with cerebral ischemia reperfusion[3]. Inhibition of inflammatory response and limit the action of free radicals on the neurons are considered as important approaches in surviving the brain cells[4], and reduction of ischemic area. Antioxidant agent may play a significant role in reduction of infarction size and tissue injury [5].

Methionine is an essential amino acid, body need it for many physiological pathways like synthesis of glutathione which is considered as more important endogenous antioxidant molecule [6]. Methionine found in two enantiomer (L- Methionine and D-Methionine) L-Methionine is the active form[7]. Methionine has an active metabolite s -adenosyle methionine (SAM) which has a significant role as anti-oxidant. SAM is a donor of methyl-group required for the more than 200 known methyltransferases encoded in the human genome[8]. The present study was designed to assess the possible neuroprotective activity of L-Methionine in cerebral I/R injury after bilateral common carotid artery occlusion (BCCAO) in rats.

MATERIALS AND METHOD

A total of 24 Adult Sprague-Dawley rats weighing (150-220 g) were purchased from Animal Resource Center, College of Veterinary Medicine- University of Kufa. They were housed in the animal house of Faculty of Pharmacy, University of Kufa, Iraq, in a temperature-controlled (25°±1C) room (humidity was kept at 60–65%) with alternating 12-h light/12-h dark cycles and were allowed free access to water and chow diet until the start of experiments. After the 1st week of localization the rats were distributed randomly into 4 groups as follow.

Group 1: sham group, rats underwent the same anesthetic and surgical procedures for an identical period of time, but without 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: One hour before surgery, rats received intraperitoneal **(IP)** normal saline (0.9% NaCl) (1 ml/kg/day) **[(9)].** Then, anesthesia and surgery with bilateral

common carotid artery occlusion (BCCAO) for 30 min. and later reperfusion for 1 hour. **Group 4: treated group,** rats received intraperitoneal L-Methionine **(IP)** 1 hour before Ischemia in a dose of (100 mg/kg), then anesthesia and surgery with bilateral common carotid artery occlusion (BCCAO) for 30 min. and later reperfusion for 1 hour.

Ischemia/reperfusion model

Rats were maintained at approx. 37°C under a light bulb and under general anesthesia ketamine & xylazine (80mg/kg & 5mg/kg intraperitoneal[10]. 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 vascular clamp and clamped for 30 min. In the reperfusion, the clamps were removed after ischemia and reperfusion was allowed to take place for 1 hour.

Sample collection:

After the period of reperfusion the brains were removed and washed out with normal saline and refrigerated for 5min in deep freeze in order to prepare it to histopathological study and TTC stain.

Tissue preparation for histopathology

Coronal brain sections were fixed with 10% formalin and embedded in paraffin wax and cut into longitudinal section of 5µm thickness The sections were stained with haemotoxylin and eosin dye for histopathological observation **(11).**

Histological analysis and damage scoring of brain

The histological observations (evaluated by a pathologist using a double-blind method) were scored using a pathological scoring scale. The following scores were used to assess the histopathological damage: Score 0, no damage; score 1 (mild), interstitial edema and focal necrosis; score 2(moderate), diffuse brain cell swelling and necrosis; score 3(severe), necrosis neutrophil infiltration and the capillaries were compressed; and score 4 (highly severe), widespread necrosis, neutrophil infiltration, capillaries compressing and hemorrhage[12].

TTC staining (For staining by the immersion method)

TTC stain is a 2, 3, 5-triphenyltetrazolium chloride commonly used to measure the infarct area in both mice and rats (10). TTC was dissolved in phosphate buffer saline (PBS) (0.2 M Na2HPO4 and 0.2 M NaH2PO4, pH 7.4-7.6), with 37°C at 2% (w/ v) concentration and used immediately for staining brain slices [13].

TTC solusion was prepared immediately before use. The sections were put in a glass petri dish containing a shallow layer of 2% TTC, and glass cover slips wetted with the TTC solution were placed on top of each slice**.** To ensure even staining, the top and bottom surfaces of the section were in contact with the glass. The dishes were covered with

aluminum foil, to prevent exposure to light because TTC is light sensitive**,** and incubated at 37°C for 30 minutes**.** The TTC solution was then replaced with 10% buffered formalin (phosphate-buffered formalin, PBF)**.** To prevent distortion and fixed, brain slices were kept flat in the Petri dish or immersion in 10% phosphate-buffered formalin (PBF) overnight as reported by Bederson *et al.* (1986)[14]. 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. Then the cerebral infarction area was observed and compared between L-Methionine treated group and control groups.

RESULTS AND DISCUSSION

L-Methionine significantly reduced the tissue injury as compared with a control and control vehicle group as shown in the histopathological studies and TTC stain. The sections below show significant brain tissue injury after ischemia reperfusion in control and control vehicle groups in compare with the sham group. The histopathological scoring of this damage was ranging between *score 3* and *score 4*. This indicates sever to highly sever injuries in both control and control vehicle groups. On the other hand, TTC staining of tissues taken from control and control vehicle groups show sever injury with a large pale infarcted area as compared with a sham group.

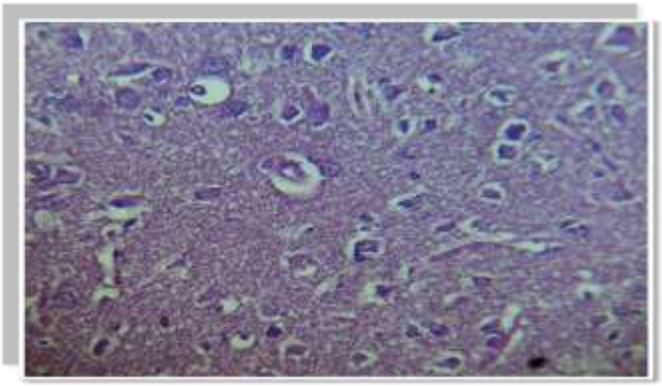

Figure 1: A Photomicrograph section of normal rats brain shows the normal tissue, and the histopathological score =0, normal. The section stained with H&E (X 40).

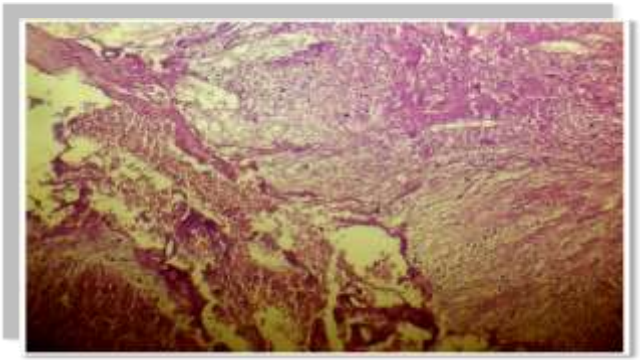

Figure 2: A photomicrograph section of brain tissue shows a moderate global ischemia with hemorrhage and *score 2* histopathological grade. The section stained with H&E (X 10).

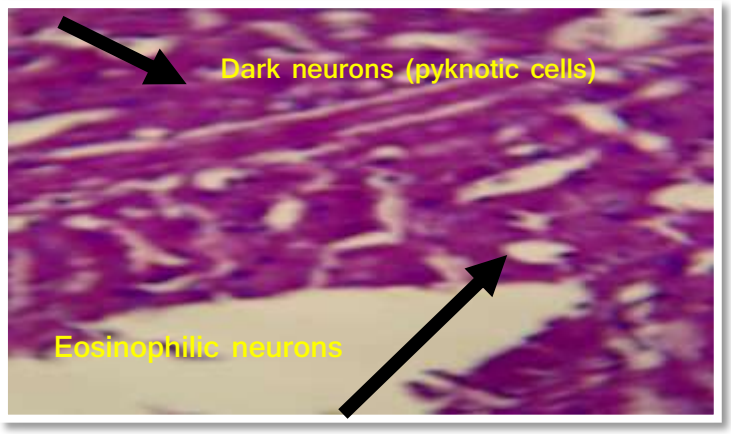

Figure 3: Photomicrographs of rat's brain section of Hypoperfusion/reperfusion in global ischemia showing eosinophilic neurons, dark neurons (pyknotic cells) and the histopathological score = 1 Slight injury, the section stained with H&E (X 40).

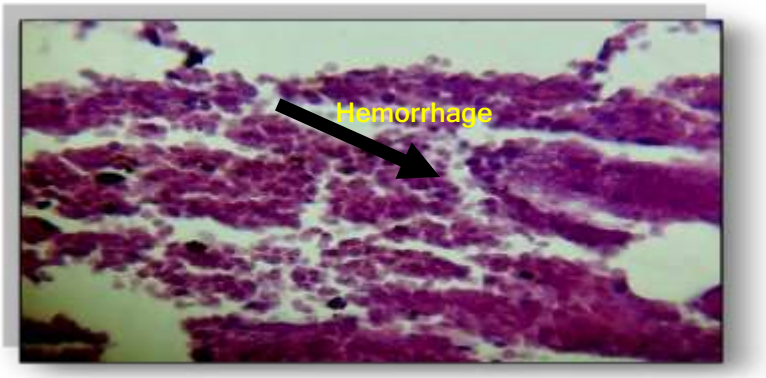

Figure 3: Photomicrographs of rat's brain section of Hypoperfusion/reperfusion in global ischemia showing necrosis and hemorrhage, and the histopathological score = 3 .The sections stained with H&E (X 40).

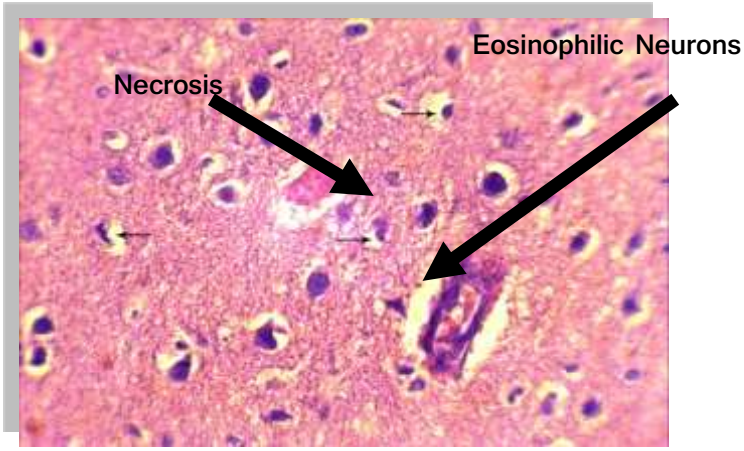

Figure 4: Photomicrograph for brain tissue of rats treated with L-Methionine showing slight edema and eosinophilic neurons, and the histopathological score =1 .The section stained with H&E (X 40).

Figure 4: Photograph of brain slide of sham group stained by TTC stain showing normal brain no cerebral infarction.

Figure 5: Photograph of coronal brain slice of control group stained by TTC stain showing significant cerebral infarction area.

Figure 6: Photograph of coronal brain slice of control-vehicle group stained by TTC stain showing cerebral infarction area.

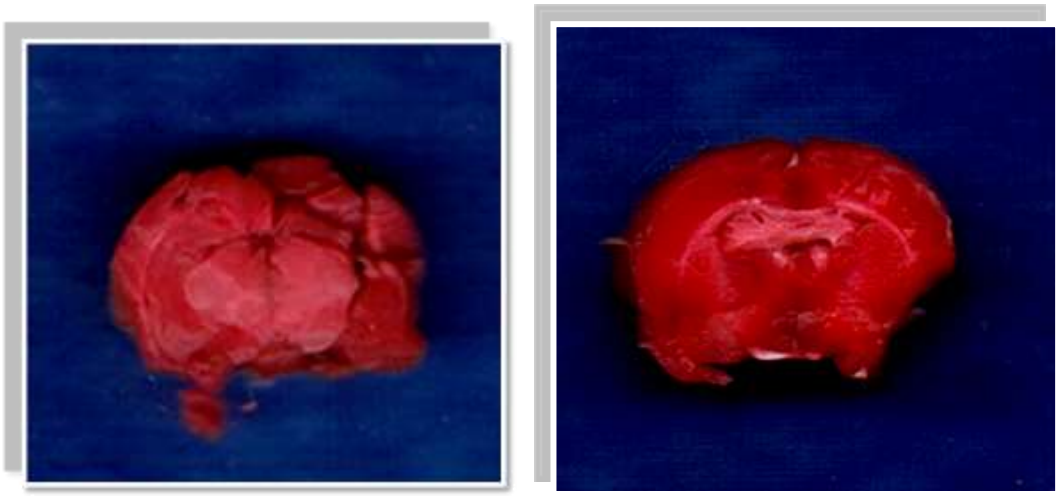

Figure 7: Photograph of coronal brain slice treated with L-Methionine stained by TTC stain showing significant decrease in cerebral infarction area.

The score of the control group shows sever cerebral injury and moderate injury. Shah *et al.* (2005) showed that in MCA/BCA occlusion for (30 min.) and then following reperfusion for (1 hour.), caused marked congestion of blood vessels [(15)]. These effects were further augmented following reperfusion i.e. Lymphocytic proliferation and neuronal necrosis. Chandrasekhar *et al.* (2010) 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 [11].

In the present study, detection 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 (induced- untreated) group as compared to L-Methionine treated group. These results are in line with Chandrasekhar *et al.* (2010); Prakash *et al.* (2011) and Lapi *et al.* (2012) [11, 16, 17]. TTC is a water-soluble dye that is reduced to formazone by the enzyme succinate dehydrogenase and cofactor NAD, present in mitochondria and stain viable tissue deep red in colour. Ischemic tissue with damaged mitochondria remains unstained (Bederson *et al.,* 1986).Methionine is a good antioxidant agent and has cytoprotective properties [14].

In this study methionine cause significant reduction in infarction size and histopathological studies as compared with control group and this comes in agreement with the results of a study that showed that S-adenosyle Methionine, (SAM, active metabolite of methionine) has a good antioxidant action in brain tissue that had subjected to experimental ischemia-reperfusion [18].

In another study they also found that methionine is very effective in preventing ischemia-reperfusion-induced myocardial necrosis where the results of their study comes in agreement

with our findings regarding both the effect ischemia reperfusion on brain tissues as well as the cerebroprotective effect of L-methionine [19]. It could be concluded from these findings that L-methionine may exerts a potential cerebroprotective effect and further measurements of more objective inflammatory parameters may confirm this conclusion.

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