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Neuroprotective Effect of Empagliflozinon Cerebral Ischemia/Reperfusion Injury in Rat Model

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Abstract

Background: Stroke is a highly troubling disorder with a high mortality rate, it is considered as a second predisposing cause of death and deficit wide word. Restriction of cerebral blood flow can disturb cellular homeostasis due to insufficient oxygen and nutrient delivery. However, the re-establishment of cerebral blood flow can aggravate the impairment of ischemic brain tissue contributing to a series of oxidative, inflammatory events resulting in cerebral ischemia-reperfusion (CI/R) injury, which eventually results in neuronal death and neurological disability.**Method:** An experimental model of 30 Sprague-Dawley rats were randomly allocated to five groups, sham group, I/R group, I/R+(DMSO as a vehicle), I/R+ intraperitoneal (i.p) Empagliflozin 5mg/kg 1 hour before induction of BCCAO, and I/R+intraperitonealEmpagliflozin 10mg/kg 1hour before induction of BCCAO.**Results:**histopathological examination showed that Empagliflozin ameliorated the histological lesions induced by CI/R. Besides, the assessment of cerebral infarction size in each group showed that Empagliflozin reduced the infarction size in both used doses in the study.The immunohistochemical analysis of cerebral tissue expressed that CI/R causesa reduction in neuronal nuclear protein (NeuN protein) in the control and control-vehicle group as compared to sham group,while pre-treatment with Empagliflozin caused re-appearance of NeuN protein in both doses groups.**Conclusions**: The study concluded that Empagliflozin has a neuroprotective effect by reduction of cell necrosis and apoptosis due to inhibition of different mechanisms that cause cerebral tissue damage and neuronal death.

Keywords: cerebral ischemia/reperfusion CI/R, Empagliflozin, infarction size,NeuN protein, neuroprotection.

INTRODUCTION:

Stroke is a highly troubling disorder with a high mortality rate. It is considered as a second predisposing cause of death and deficit wide word.Two types of stroke, either hemorrhagic or ischemic, the latter is the most common type accounting for 87% of all cases of cerebrovascular accident [1]. The ischemic stroke(brain infarction) result from occlusion of

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the cerebral vasculature by either thrombus or emboli leading to depriving the neuronal cells of oxygen and nutrients needed for their vitality and normal functions[2], while the other type (hemorrhagic stroke)is caused by intracranial hemorrhage, although this stroke subtype accounts for only about 15% of all stroke cases it's the highest mortality rate with no available treatments yet [3].Ischemic stroke is either hereditary which is about 37.9%[4] or due to 10 modifiable risk factors that form about 91.5% of the population attributable risk of ischemic stroke globally, these factors include high blood pressure history(≥160 /90 mmHg), sedentary lifestyle, high waist to hip ratio, smoking, alcohol consumption, diabetes mellitus, hyperlipidemia, atrial fibrillation, diet, psychosocial stress and depression[5]. The immediate therapy of acute ischemic stroke is highly recommended. The patient is preferred to be treated within 4.5 hours of stroke onset with an intravenous plasminogen activator[6] or mechanical thrombectomy of large vessels within 6hours of stroke onset[2]. Although they are very important, both strategies are time-critical and several attempts were made to increase the benefits from them. [5]. Furthermore, the prophylactic prevention of stroke is very important and can be achieved by the treatment of cardiovascular causes such as hypertension, atherosclerosis, and the use of antithrombotic drugs. [5]. Ischemic stroke is accomplished by complex path physiological events that begin with failure in energy production, acidosis, loss of cellular homeostasis, excitotoxicity, neuron, and glial cell activation, and BBB disruption by infiltrated leukocytes[7].The reperfusion is like ischemia also can cause a deleterious effect on brain parenchyma [8] causing what is called reperfusion injury[9].The reperfusion can initiate several inflammatory processes that exacerbate the damage to the cerebral vasculature and the adjacent brain tissue through activation of microglia and astrocyte as well as recruitment of circulating Neutrophils, these cells release a neurotoxic molecule such as cytokines, nitric oxide, ROS, and matrix metalloprotease which disrupt BBB and cause delayed neuronal death[10]. Ischemic insult is followed by a disturbance in the microcirculation in the ischemic territory. These disturbances begin to develop within the first hours of cerebral ischemia and persist despite the re-establishment of reperfusion [11]. Within minutes of cerebral ischemia occurrence, the core of brain tissue that is exposed to the most dramatic blood flow limitation subjects to fatal injury, and is then its cells undergoes necrosis[12]. This dead nucleus is surrounded by an area of less affected tissue that becomes functionally inefficient by decreasing blood flow but remains active in metabolism, this border region - known as " penumbra" - may form up to half of the total lesion volume during the initial stages of ischemia and form a salvageable area after stroke therapy[12-13].Brain tissue injury is progressed by major four mechanisms including inflammatory responses, oxidative stress, BBB disruption, and neuronal apoptosis, these processes induced damage may take a period from hours to days.Empagliflozin (formerly known as BI 10773)[14] with a chemical formula of (1-chloro-4-[b-D-glucopyranos-1-yl]-2-[4-([S]-tetrahydrofuran-3-yl-oxy) benzyl]-benzene, is a highly selective sodium-glucose cotransporter-2 (SGLT-2) inhibitor which administered orally and reduces blood sugar by inhibition of glucose reabsorption in renal tubules in people with type 2 diabetes[15]. Its mode of action is exceptional, as it is independent of the extent of insulin sensitivity or the activity of pancreatic β-cells[16]. More recent studies have identified substantial cerebral SGLT2 expressions in reaction to neurological insults[17], this suggests a potential therapeutic action of Empagliflozin in brain disorders as a selective SGLT2 inhibitor.

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MATERIALS AND METHOD

The study has been approved by the central committee for the animal ethics of KufaUniversity. A sample of 30 adult Sprague-Dawley rats (Chandrashekhar et al., 2010) weighing 250-300 g have been purchased from the National Center of Research and pharmaceutical control. They have been housed in a temperature-controlled (25°±1C) room in the Kufa College of Science Animal House (humidity was kept at 60-65 percent) with alternating 12-h light/12-h dark cycles and the rats have been allowed free access to water and chow diet before experiments began. The rats randomly distributed into five groups after the first week of optimization as follows: (sham group): the rats in this group have been subjected to anesthesia and surgical process without undergoing bilateral common carotid artery occlusion BCCAO.(Control group): the rats in this group have been anesthetized and subjected to BCCAO for 30min. followed by a reperfusion period of 1hour.(vehicle group)**:**in this group, the rats have been injected by DMSO intraperitoneally 1hour before performing BCCAO for 30min. followed by 1hour of reperfusion.(treatment group-low dose): the rats of this group were injected with 5mg/kg of intraperitonealEmpagliflozin 1hour before BCCAO for 30min., then reperfusion for 1hour was allowed. (Treatment group-high dose): The rats in this group have been injected intraperitoneally with 10mg/kg 1hour before 30min. of BCCAO, then reperfusion has been allowed for 1hour.

Preparation of the drug: Empagliflozin (CAS: 864070-44-0) has been purchased from **MedChem Express Co. USA.** The dose has been prepared immediately before use by dissolving each 5mg of the drug in 1ml of DMSO[18]and administered in a dose of 5mg/kg intraperitoneally (I.P) to the low dose group animals(Michel et al., 2015a, Nair and Jacob, 2016) and 10mg/kg I.P to the high dose animal group[19]. Both groups have been administered Empagliflozin 1 hour before the induction of I/R[20]. The animals in the control vehicle group were injected with equal amounts of (DMSO) i.p also 1-hour before induction of global I/R.

Induction of global ischemia: Global ischemia Induced by bilateral common carotid artery occlusion BCCO[21]. Rats have been preserved at approximately 37°C behind a lamp and under general anesthesia with ketamine and xylazine (80 mg/kg & 5 mg/kg intraperitoneally)[22]. Animals in the supine posture have been positioned on the back; the lower and upper limbs were fixed with plasters. A short median incision was produced in the neck and the two carotid arteries were isolated from the vagal nerves, then shown bilaterally and obstructed using vascular clamps and locked for 30 min. After that, the clamps have been removed to permit reperfusion for 1hour[23].

Samples preparation: The rats have been euthanized immediately to extract the brains and prepared for sampling. The brains of each rat were washed with cold phosphate buffer solution and placed in an ice container, each brain sliced into two coronal sections, the first has been kept in 10% formalin for later histopathological and immunohistochemical tests.The second section has been frozen at -20 for 20 minutes before additional slicing into more equal coronal sections (2mm) for TTC staining.

Tissue sampling for histopathology: First, the brain coronal sections have been set in 10% formalin, and then inserted into paraffin wax. For histopathological analysis preparation, they have been cut down to 5 μm with the help of a section cutter and stained with Hematoxylin and Eosin (H&E) stains[24].

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Histological findings (examined by a pathologist that used a double-blind method) have been graded using a scale of pathological scoring as follow[25]:
- Zero (normal): no morphological signs of potential harm.
- 1 (slight): edema or eosinophilic or darkened neurons (pyknotic) or dark/ shrunk cerebella Purkinje cells.
- 2 (moderate): at least two minor hemorrhages.
- 3 (severe): distinct infarction foci (local necrosis).

Measurement of infarction size by TTC staining: The infarction area has been measured by (immersion method) using 2, 3, 5triphenyltetrazolium chloride (TTC) stain**.** The salt of tetrazolium is reduced to a red-colored formazan substance by dehydrogenises found in the mitochondria so that viable tissue has stained red while infarct tissue has stayed pale[25].Brain tissue slices have been stained with TTC stain prepared instantly at a concentration of 0.2 % (w/ v) in PBS before brain slicing. After the brains split into 2mm thick coronal slices, it soaked in TTC stain at 37 ° C for 30 min in a glass petridish which covered by aluminum foil to avoid the effect of light on TTC stain because it is photosensitive. Then the slices have been washed twice with PBS, at the end, the sliceshave been placed in 4% buffered formalin at the transparent dish and photographed by digital camera. The images have been analyzed by using (image J)software to measure the infarction area where the rad area represents the viable tissue, while the pale represents the infarct tissue which calculated as a percentage, then the infarction areas between the experimental groups have been compared[26]. The infarct area has calculated by the following equation[27]:

Infarct area %=(vc-vl)/vc x100

Where:

Vc is the control hemisphere volume

Vl is the lesion hemisphere non-infarct tissue volume.

Measurement of NeuN protein

The brain tissues obtained from each group have been applied to measure the percent of cells labeled by the NeuN antibody using immunohistochemistry technique, as instructed by manufacturer protocols. We use Elabscince laboratory NeuN/BRfox3 polyclonal antibody.The Immunoreactivity has been graded by determining the percentage (P) of positive cells displaying distinctive staining (from an unnoticeable amount or 0 % to highly sensible staining or 100%) and by assessing the staining strength (I) (1, weak staining; 2, moderate staining; and 3, strong staining).Then the scores were graded by multiplying the staining intensity (I) by the percentage of positive cells(P) using the quick score Q equation[28]:

Q = p x I,maximum Q=300

Where:

I is the staining intensity.

P is the percentage of positive cells.

STATISTICAL ANALYSIS

Data have been collected and included in a data-based system and analyzed by a statistical package of social sciences ((SPSS, Inc., Chicago, IL, USA)) version 24. Parametric data have been expressed as mean ± standard error of mean (SEM). It has been analyzed statistically

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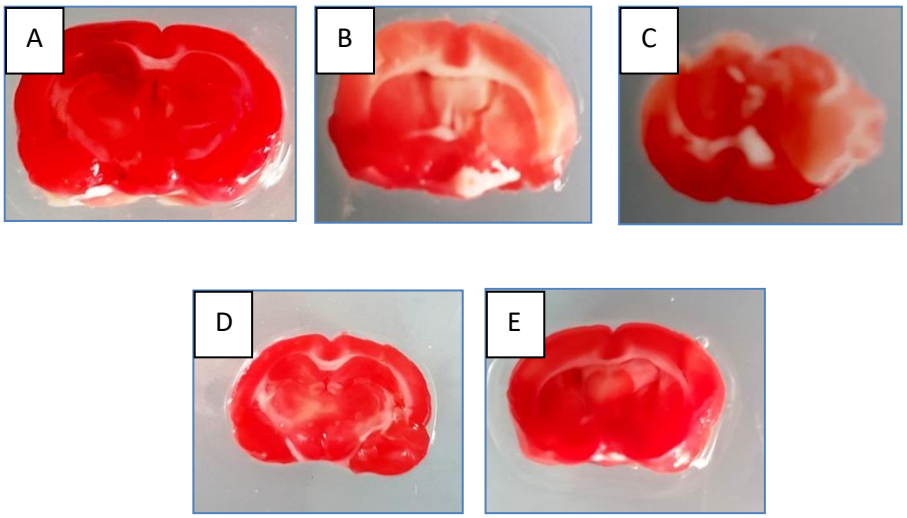
using student t-test and ANOVA post Hoc tukey test to evaluate multiple comparisons between groups. For comparison between histopathological scores in two groups, Mann-Whitney U- test has been performed as a non-parametric test. In all tests, when the value of $p>0.05$ statistical significance is considered.

RESULTS:

Empagliflozin reduces cerebral infarction size

Analysis of rat brain coronal sections photographs using (image j) software after tri-phenyl tetrazolium chloride (TTC) staining demonstrated that cerebral I/R increased in the percentage of cerebral infarction size significantly ($p=0.001$) in control group relative to sham group (23.5±0.62 vs. 0), while it's not showed significant difference($p=1$) in infarction size between control and control vehicle groups (23.5±0.6 vs23.38±0.4). Pre-treatment with Empagliflozin showed that infarction size reduced significantly in both low ($p=0.001$) and high dose Empagliflozingroup ($p=0.002$) in comparison to control vehicle group (10.76±0.5 vs23.38±0.4) and (10.04±0.5 vs. 23.38±0.4) respectively. Whoever no significant difference ($p=0.8$) in infarction size percentage between low and high dose Empagliflozingroups (10.76±0.5 vs. 10.04±0.5) as shown in figure (1).



Figure(1) infarction size percentage in each group in the study, (A) sham, (B)control, (C) control-vehicle, (D) low dose empagliflozin group, (E) high dose empagliflozin group.

Empagliflozinameliorated histopathological damage

Cerebral I/R induced cerebral damage ranged from slight to severe in control and control-vehicle groups as compared to sham group which appeared as normal tissue under the microscope. Pre-treatment with Empagliflozin caused a significant lowering in the score of histopathological damage in both low and high-dose Empagliflozin groups, while no significant difference has been shown in lesions between the two treatment groups as shown in figure (2).

Empagliflozin induced re-appearance of NeuN protein in the cerebral cells

Immunohistochemical analysis of paraffin-embedded cerebral tissue demonstrated significant reduction ($p=0.003$) in the expression score (Q-score) of NeuN protein in control group in comparison to sham group when examined under the microscope (14.16±2.03 vs.

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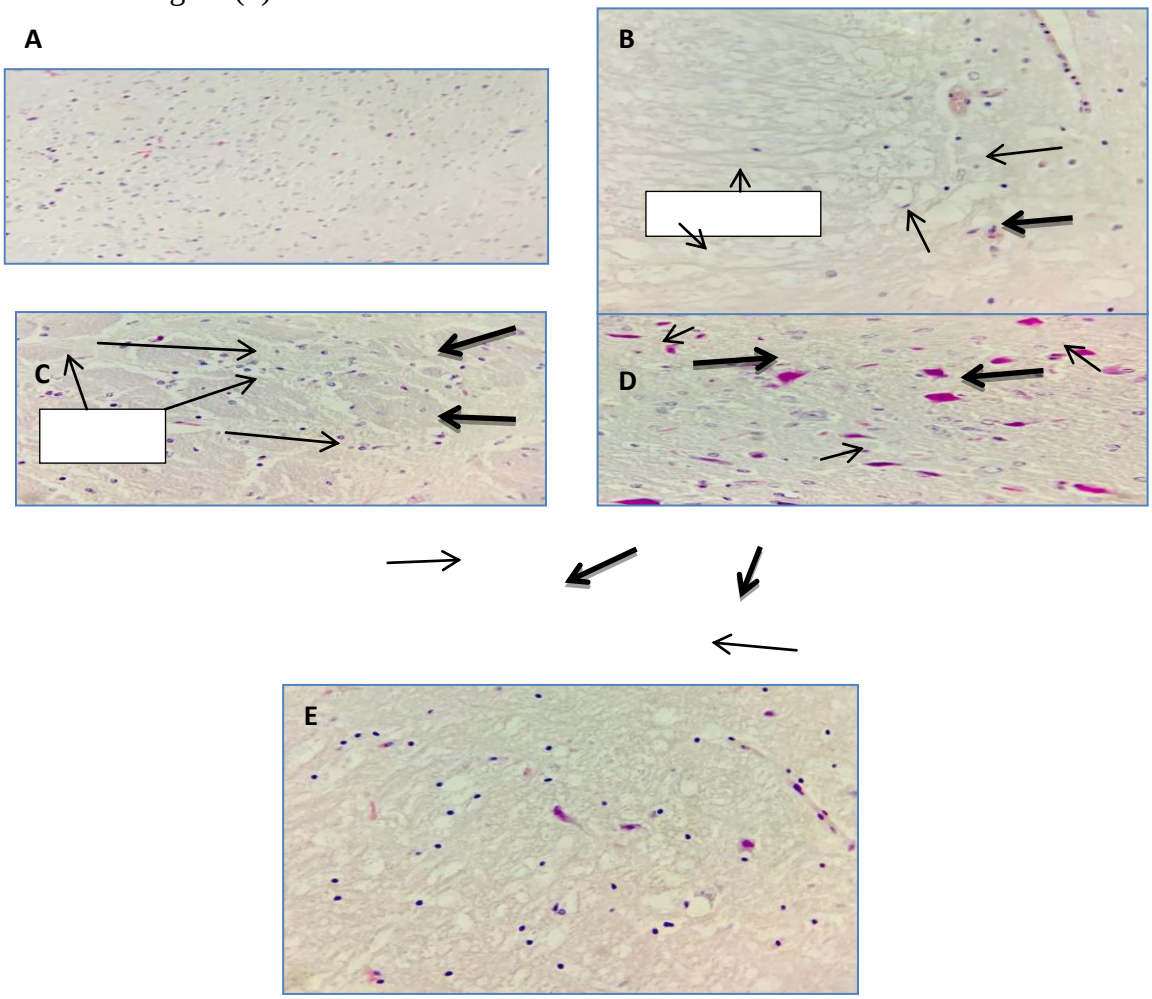
293.3±3.33), while it displayed no difference in NeuN Q-score between control and control-vehicle group (p=1). Pre-treatment with Empagliflozin exhibited re-appearance of NeuN protein in the examined tissue of both Empagliflozin groups (low dose p=0.001 and high dose p=0.002) demonstrated by increasing its Q-score in comparison to control-vehicle group (234.16±17.49 vs. 15±2.59)and (240.8±10.06 vs15±2.59) respectively, whoever no significant difference (p=0.98) in NeuN Q-score between low and high dose Empagliflozin (234.16±17.49 vs. 240.8±10.06). The changed in NeuN expression between the study groups are illustrated in figure (3).



Figure(2):histological finding after H&E staining showing (A) sham group express normal tissue appearance(x100),(B)control group shows score3 damage x400,(c)control-vehicle group shows score3 damage x400,(D)low dose empagliflzin group shows score1 damage,and(E)high dose empagliflozin group shows score1 damge.nicrosis denoted by spoted arrows,edema by thin arrowes ,and darke eosinophlic neorons by thick arrowes.

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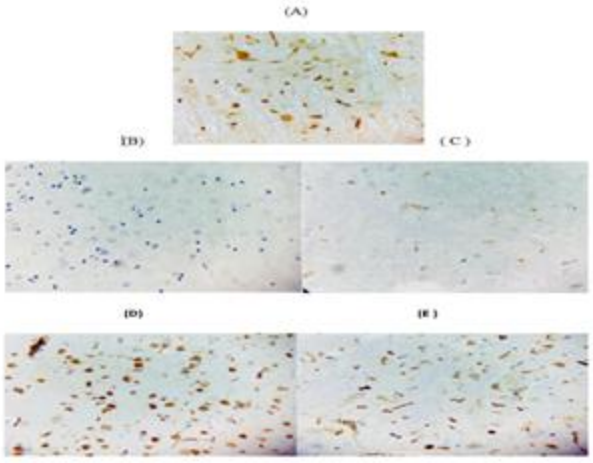

Figure **Error! No text of specified style in document.** The immunohistochemical expression of the nuclear NeuN/fox3 protein among the five experimental groups,(A)sham group showing strong staining and a high percentage of NeuN positive cells expression. (B) The control group with a negative result. (C) control-vehicle group showing scattered NeuN positive cells with moderate staining intensity. (D) Low dose Empagliflozin group showing a high percentage of NeuN positive cells and strong staining intensity. (E) High dose Empagliflozin group showing a high percentage of NeuN positive cells with strong staining intensity.

DISCUSSION

The ischemic stroke (brain infarction) results from occlusion of the cerebral vasculature by either thrombus or emboli leading to depriving the neuronal cells of oxygen and nutrients needed for their vitality and normal functions [2]. Global ischemia is the type of ischemia that affects a large area or the entire brain as a sequence of cerebral blood supply reduction under the critical threshold, it is usually transient so the patient affected can survive but may suffer from permanent neurological dysfunction. This type of ischemia is used in animal models of ischemia-reperfusion (I/R)[29].When there is an imbalance between the metabolic requirement and the delivery of oxygen and glucose the first phase of cerebral injury called "ischemic injury" occurs which accomplished by hypoperfusion depriving the brain of oxygen and glucose causing bioenergy failure by reducing the level of ATP production, this results in a disruption in ATPas dependent pumps causing high Na+ influx with K+efflux, giving arise to anorexic depolarization in the neuronal and glial cells membrane, the high Na+ influx cause increase in the osmotic inflow of water into the cell resulting in edema, cell rupture, and necrosis[7]. "Reperfusion phase" begins when the cerebral blood supply is restored to the ischemic area either by Thrombolysis or mechanical thrombectomy[30]with activation of inflammatory elements, the release of neurotransmitters, endothelial cells dysfunction that leads to disruption of BBB, edema and neurological deficit[31]. Reperfusion provokes over-production of reactive oxygen species(ROS) and reactive nitrogen species(RNS) to them the brain tissue is highly sensitive [32]. Several experimental studies in stroke models show that the cerebral tissue damage is continuing to extend despite the reperfusion therapy, this termed as ischemia/reperfusion injury[30], so many emerging

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studies are working by targeting the anti_stroke agents during the reperfusion injury [33]. In a parallel situation, Glutamate which is the major excitatory amino acid (GLU) in CNS plays a key role in excitotoxicity of the neuronal cells by increasing the intracellular Ca+ ion which inter the neuron by a voltage-gated or ligands gated ion channel causing activation of intracellular protease, lipases, kinases, and end nucleases provoking the apoptotic cell death[34].The current study explained that cerebral ischemia-induced cerebral damage reached to higher score (score3) in histopathological lesions. In resembling study, according to M. H. ShalavadiV. M. Chandrashekhar *et al.* a marked Neutrophil invasion has been observed in control, cytoplasmic space expanded and cell density reduced, cell structure modified, hemorrhage and neuronal cell death were also observed after 30 minutes of GCI and 24 hours of reperfusion[35].Whoever, these lesions we observed were showed to be ameliorated in the two Empagliflozin (5mg/kg and 10mg/kg) groups that administered the drug prior to induction of global cerebral ischemia. This observation has been revealed byEF AminRA Rifaai *et al.*explored the neuroprotectiveactivity of Empagliflozin through its anti-inflammatory and antioxidant mechanism, those researchers reported that treatment with Empagliflozin shows a substantial decrease in neuron degeneration detected in vehicle-treated I/R rats in both the brain cortex and hippocampus regions[19].In the current work,the histological findings were found to correlate with infarction size assessment results; the infarction area was reduced in Empagliflozin groups to approximately 50% as compared to control and control vehicle group where CI/R caused expanded infarction size after induction of global ischemia in comparison to sham group. The results of this study correspond with other obvious studies'results; all demonstrated that cerebral ischemia /reperfusion increases infarction size in the control group relative to sham group[23-25],where EF AminRA Rifaai et al. studied the neuroprotective effect of Empagliflozin and established the power of Empagliflozin to reduce the cerebral infarct area after 30 minutes of global cerebral ischemia followed by 24 hours of reperfusion in induced hyperglycemic rats when Empagliflozin administered 24 hours before BCCAO[19].NeuN is a specific neuronal nuclear antigen that discovered in 1992, it is demonstrated in mature neurons of approximately all vertebrates and expressed in specific developmental stages which indicate terminal neuronal differentiation and termination of its cell cycle (post-mitotic)[36].During the last two decades, NeuN protein was considered a faithful mature neuronal marker[37].NeuN was commonly used in stroke studies because it is a specific and accurate marker of mature neurons. On the first hand, NeuN's expression level was used to specifically determine neuronal death or loss[38]. On another side, the re-appearance of NeuN-positive cells in experimental therapeutic studies has been a valid marker for quantifying the therapeutic effects of agents[39].In the present work, NeuN is used as an indicator of neuronal cell survival. The immunohistochemical analysis exhibited full expression of NeuN protein in sham group with a significant reduction in its expression to dis-appearance in control and control-vehicle group. Stroke experiments have showed that NeuN-positive cells in disease-related centers decrease to a greater degree than in normal areas, and this effect has been indicated to be caused by neuronal death or destruction [40-41]. Our study is consistent with M avoliJ. Fourtounis *et al.* who explained that the loss of NeuNimmunoreactivity reflects neuron cell death in ischemic damaged brain area as the result of NeuNimmunoreactivity compared with the results of TUNEL staining that also showed increase apoptotic death in the same area[41]. Besides, Unal-cevikM

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Kilinc*et al.* showed that NeuN-positive neurons in MCAO rats model decreased by 27 percent in the penumbra and 62 percent in the ischemic core region[42]. A rat model of GCI by K SadelliJC Stamegna *et al.*showed a massive reduction in the NeuNimmunoreactivity in CA1 of the dorsal hippocampus after I/R indicating neuronal cell death and reduction in the number of viable cells in this region [43],according toP-Y WenJ. Li *et al.* findings in a model of 2 hours of focal ischemia followed by 24hours reperfusion observed a significant drop in the immunostaining of NeuN positive neurons as compared to sham groups, these studies were highly correlated to the present study[44]. The current study showed re-appearance of NeuN protein in both treatment groups, it showed higher immunoreactivity with its antibody relative to control and control-vehicle groups indicating the neuroprotective effect of Empagliflozin and correlation with the findings obtained from histopathological and infarction size analysis.A recent study by C. Hierro-BujalanceC. Infante-Garcia et al. assessed the effect of Empagliflozin on NeuN protein expression in a mice model of AL-Alzheimer and diabetes. Empagliflozin showed to increase cortical NeuN/DAPI ratio indicating an improvement in pathological processes concerning both Alzheimer's and diabetes[45].

CONCLUSIONS

The study concluded that Empagliflozin has a neuroprotective effect as it can lower the sign of cerebral tissue damage and reduce the sign of apoptotic and necrotic cell death.

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