Anti-inflammatory and antioxidant effect of Empagliflozin on cerebral ischemia/reperfusion injury in rat model

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Abstract: Background: 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+intraperitoneal Empagliflozin 10mg/kg 1hour before induction of BCCAO. The brain tissue levels of IL-1β, ICAM-1, and F2-isoprostane were measured in each group in our study. Results: The two doses of (5mg/kg and 10mg/kg) Empagliflozin were significantly reduced the brain tissue level of IL-1β, ICAM-1, and F2-isoprostane as compared to I/R and I/R+vehicle groups.Conclusions: From the results above we concluded that Empagliflozin has a neuroprotective effect seeing that it's anti-inflammatory and anti-oxidant activity.

Keywords: Ischemia-reperfusion injury I/R, Bilateral common carotid artery occlusion BCCAO, Empagliflozin,interleukin-1β (IL-β), intercellular adhesion molecule-1 (ICAM-1), F2-isoprostane.

1. Introduction

Stroke is a very morbid disorder with a high mortality rate; it is considered a second predisposing cause of death and deficit wide word [1]. It causes the annual death of about 5.5 million people with about 44 million disabilities [2-3]. The stroke is either hemorrhagic or ischemic which accounts for 87% of all cases of cerebrovascular accidents[4]. 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 [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 blood-brain barrier disruption by infiltrated leukocytes [6]. The immediate reperfusion is intended to re-establish the cerebral blood supply carrying oxygen and glucose to the ischemic area, achieved either by Thrombolysis or mechanical thrombectomy [7]. Unfortunately, it forms another cause for further cerebral damage due to an increase in the delivery of inflammatory elements to the injured area [8]. Reperfusion provokes overproduction of reactive oxygen species (ROS) and reactive

nitrogen species(RNS) to them the brain tissue is highly sensitive[9]. They released from activated microglia, damaged neurons, and infiltrated Neutrophils[10] playing a key role in I/R induced brain damage by induction of intracellular calcium accumulation and induces necrotic or apoptotic cell death due to impairment of mitochondrial permeability transition pores MPTP[11]. ROS also implicated in the activation of macrophages and other immune cells causing further release of ROS amplifying the cerebral damage [12]. Oxidative stress is also implicated in the development of inflammatory response after I/R through the stimulation of gene transcription of some pro-inflammatory factors including the NF-κB, IRF1, STAT3, and HIF-1α. [13]. Isoprostane are predominant products of free radical-induced peroxidation of membrane phospholipid arachidonic acid that independent of cyclooxygenases[14]. The inflammatory reaction is highly implicated in the progression of I/R injury. the early response is achieved by the resident immune cells(microglia)which is activated in response to ischemia to has function of reactive species releasing, phagocytosis, antigen presentation, and production of pro-inflammatory mediators such IL-1β, TNF-1α, IL-6, and MMP[15] and recruitment of the circulating Neutrophils to the site of ischemic injury[16]. Cytokines are small non-structural proteins, they have also named lymphokines, monokine, chemokine, and interleukins (IL)[17] play an important role in the progress of stroke-related inflammation. They promote the development of adhesion molecules, attract leukocytes, and trigger thrombogenesis during brain ischemia through an increased activation factor for platelets plasminogen-activator inhibitor-1, and tissue factor[18]. ICAM-1 is the primary molecule of endothelial cell adhesion which mediates leukocyte constant adhesion and consequential leukocyte transmigration[19]. ICAM-1 is expressed at low levels in endothelial cells, leukocytes, epithelial cells, and fibroblasts[20]. ICAM-1 mRNA expression increased immediately after the exposure to ischemia or induced by pro-inflammatory cytokines in an NF-kβ-dependent way [20]. Empagliflozin(formerly known as BI 10773)[21] 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[22]. Its mode of action is exceptional, as it is independent of the extent of insulin sensitivity or activity of pancreatic β-cells[23].In addition to the hypoglycemic effect, Empagliflozin has numerous beneficial therapeutic effects as it has natriuretic, uricosuric, in addition to established cardioprotective and nephroprotective effects. Empagliflozin had previously been confirmed to be associated with a marked decrease in cardiovascular morbidity and death according to (EMPA-REG OUTCOME)[24]. A study on type 2 diabetic induced rats found that Empagliflozin can reduce oxidative stress in aortic vessels and inhibit endothelial dysfunction in the aortic rings [25] suggesting it's antioxidant activity. Empagliflozin treatment for 8 weeks in mice model showed an anti-atherosclerotic effect in aortic arch/valve with a reduction in plasma level of IL-6, TNF-α1, and MCP-1 confirming its anti-inflammatory action [26]. Our study aimed to assess the neuroprotective effect of Empagliflozin through its inflammation and oxidative stress ameliorating mechanism.

2. Materials and method

The study was approved by the central committee for the animal ethics of Kufa University. A sample of 30 adult Sprague-Dawley rats [27] weighing 250-300 g were purchased from the National Center of Research and pharmaceutical control. They were 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 were allowed free access to water and chow diet before experiments began. The rats were randomly distributed into five groups after the first week of optimization as follows: **(sham group):** the rats in this group, subjected to anesthesia and surgical process without undergoing bilateral common carotid artery

occlusion BCCAO.**(control group):** the rats in this group, anesthetized and subjected to BCCAO for 30min. followed by a reperfusion period of 1hour. **(Vehicle group):** in this group, the rats were 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 intraperitoneal Empagliflozin 1hour before BCCAO for 30min., then reperfusion for 1hour was allowed. **(Treatment group-high dose):** the rats in this group were injected intraperitoneally with 10mg/kg 1hour before 30min. of BCCAO, then reperfusion phase of 1hour.

Drug preparation: Empagliflozin (CAS: 864070-44-0) was purchased from **MedChem Express Co. USA.** The dose was prepared immediately before use by dissolving each 5mg of the drug in 1ml of DMSO [28]and administered in a dose of 5mg/kg intraperitoneally (I.P) to the low dose group animals[29-30] and 10mg/kg I.P to the high dose animal group[31]. Both groups were administered the drug 1 hour before the induction of I/R[32].

Induction of global cerebral I/R: Global ischemia Induced by common carotid artery occlusion BCCO [33]. Rats were preserved at approximately 37 ° C behind a lamp and under general anesthesia with ketamine and xylazine (80 mg/kg & 5 mg/kg i.p)[34]. Animals in the supine posture were positioned on the back, the lower and upper limbs were fixed with plasters. A slight 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 were removed to permit reperfusion for 1hour [18]. After the hour of reperfusion, the rats were sacrificed, and the brains were isolated for ELISA and immunohistochemical analysis.

Measurement of IL-1β and F2-isoprostane: IL-1β and F2-isoprostane (8-iso-prostaglandin) cerebral tissue concentrations were measured using (bioassay technology laboratory, shanghai. china) ELISA kits. The brain tissue samples were ground into small particles and mixed with homogenization solution containing 0.2% cocktail protease inhibitor and triton- X100 in ice-cold phosphate buffer and homogenized using ultrasonic liquid processer followed by centrifugation at 2000 RPM for 30min.at 4°c. The supernatant was collected and utilized to measure IL-1β and F2-isoprostane concentration occurring to the manufacturer protocols of these kits.

Measurement of cerebral ICAM-1 level: Brain tissue samples were placed in 10% formalin and embedded in paraffin wax to be prepared for immunohistochemical measurement of ICAM-1 expression using Elabscince ICAM-1/CD54 polyclonal anti-body. The immunohistochemical analysis was performed according to the manufacturer's protocol. ICAM-1 was expressed as Q-score by multiplying the intensity of staining with the percentage of ICAM-1 positive cells [35].

Statistical analysis

Data were 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 were expressed as mean ± standard error of mean (SEM). It was analyzed statistically using student t-test and ANOVA post Hoc tukey test to evaluate multiple comparisons between groups. In all tests, when the value of $p \geq 0.05$ statistical significance is considered.

3. Results:

Cerebral I/R induced a marked elevation in the level of inflammatory mediators IL-1β and ICAM-1, in addition to increasing F2-isoprostanes, a product of oxidative stress-induced cellular damage. SO the inflammatory mediator IL-1β cerebral concentration was significantly (p=0.001) elevated in the control group in comparison to the sham group (984.5±11.4 vs. 245.5±1.4 ng/L), while we observed an insignificant difference (p=0.997) in IL-

1β concentrations between control and control vehicle group (984.5±11.4 vs. 976.5±16.1ng/L). Pre-treatment with each dose of Empagliflozin (5mg/kg and 10 mg/kg) caused a significant lowering (p=0.001 and p= 0.002 respectively) in the cerebral concentration of IL-1β relative to the control-vehicle group(306.2±20.3 vs. 976.5±16.1ng/L)and(266.7±21 vs. 976.5±16.1ng/L). There was no significant difference(p=0.409) in cerebral concentration of IL-1β between the low dose and high dose Empagliflozin groups, the difference in the concentrations of IL-1β among study groups shown in figure (1), also. The immunohistochemical analysis of cerebral ICAM-1 showed a high expression score of this inflammatory marker in the control group with moderate staining intensity relative to sham group brain tissue samples which showed weak staining intensity with very low to a negative expression of ICAM-1(P=0.001),(96.66±5.573 vs. 9.16±1.539). Empagliflozin pre- treatment ameliorated cerebral ICAM-1 expression significantly in both low dose and high dose treatment groups (p=0.001, 0.003 respectively) as compared to the control vehicle group (10.8±3.584 vs. 98.3±7.494) and (10±3.898 vs. 98.3±7.494). Where there was no difference in ICAM-1 expression score between the two Empagliflozin groups (p=1), the change in Q-score of ICAM-1 between the study groups summarizes in figures (2) and (3). The cerebral concentration of F2- isoprostane (8-iso-prostaglandin f2α) as a marker of oxidative stress was elevated significantly (p=0.001) in the control group relative to the sham group (42.27±4.67 vs. 8.40±0.71 ng/L). Meanwhile, there was no significant difference (p=0.99) in F2-isoP concentration between control and control vehicle groups(42.27±4.67 vs. 41.25±4.80 ng/L). Empagliflozin ameliorated oxidative stress by a significant reduction of the cerebral concentration of F2-isoP in both low dose (p=0.002), and high group (p=0.003) groups when compared with the control vehicle group(14.79±1.38 vs. 41.25±4.80ng/L),(12.15±0.65 vs. 41.25±4.80 ng/L) respectively. However, low dose and high dose Empagliflozin groups did not show a significant difference in F2-isoP concentration between them(p=0.97). The change in the cerebral level of F2-isoP between the five study groups was shown infigure (4).

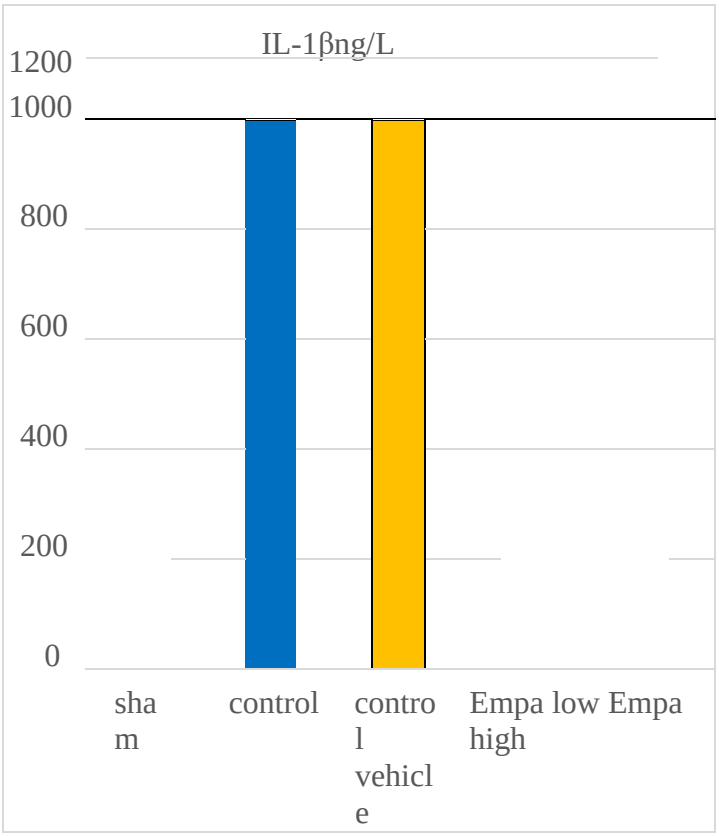

Figure (1): Cerebral concentration mean±SEM of IL-1B among the five study groups

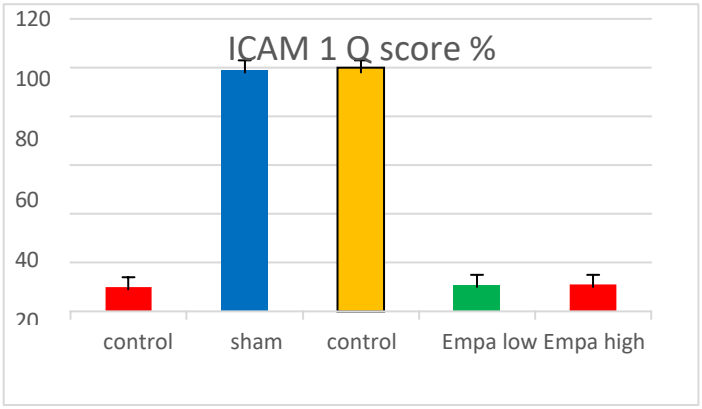

Figure (2): Q-score mean±SEM of cerebral ICAM-1 among the five study groups

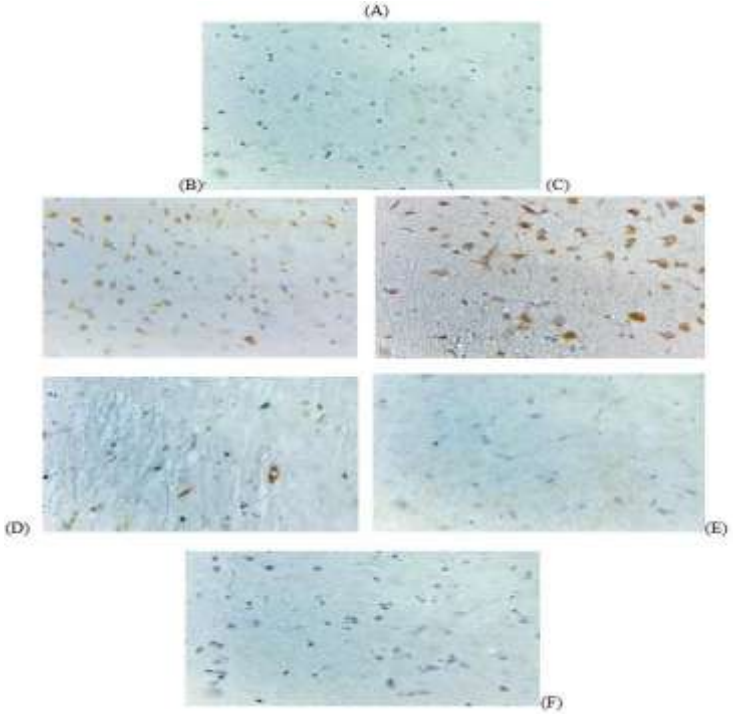

Figure (3): The immunohistochemical expression of membranous ICAM 1/CD54 in rat brain tissue in the five study groups. were (A) sham group showing negative result (no ICAM-1expression) x400,(B)control group showing a lot of ICAM-1 positive cells expression with moderate staining intensity x400,(C)control- vehicle showing a lot of ICAM-1 positive cells with moderate staining intensity x400,(D)low dose Empagliflozin group showing few ICAM-1 positive cells with moderate intensityx400,(E) another low dose Empagliflozin group showing negative ICAM-1 x400, and(F)high dose Empagliflozin group showing negative ICAM-1expression x400.

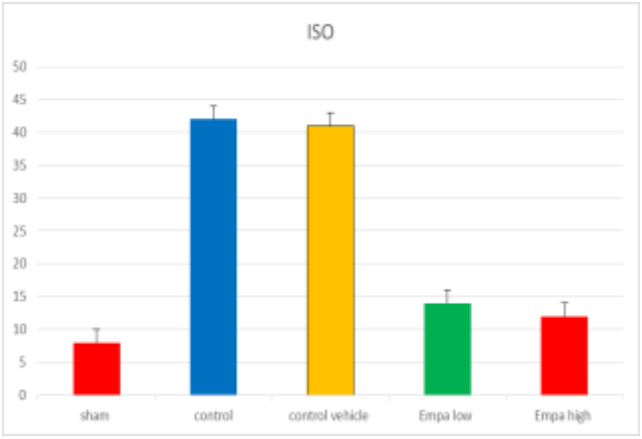

Figure (4): Cerebral concentration mean+SEM of F2-I isoprostane among the five study groups

4. Discussion

Inflammation has been involved in the pathogenesis of ischemic stroke. Inflammatory cells invade the damaged brain parenchyma as a response to brain injury and BBB destruction. Resident brain cells(microglia) are stimulated to produce pro-inflammatory cytokines such as TNF-α1, IL6, and IL-1β [36]. The pro-inflammatory cytokines have a predominant role in the development of cerebral ischemia, as they can recruit leukocytes to the site of injury; stimulate adhesion molecules synthesis in leukocytes, endothelial cells, and other cells amplifying the inflammatory process that exacerbates the damage. On the other hand, cytokines induce thrombogenesis by stimulating plasminogen activator inhibitor-1(PAI-1) tissue factor, and platelet-activating factor, while inhibiting tissue plasminogen activator [37]. IL-1β is the first among IL-1 to be expressed in response to local brain injury or stroke within 1 hour of an experimental rat model of cerebral ischemia [38]. Evidence from human cell culture indicates that hypoxia itself causes the development of IL-1β in endothelial cells, which then upregulated leukocyte adhesion molecules through an autocrine mechanism [39]. The level of IL-1β has increased dramatically in the ischemic brain relative to sham 1 day after MCAO animal model and significantly dropped after 3 days and 6 days after MCAO [17]. Many studies had approved the noxious effect of IL-1β after ischemic stroke, two studies in mice and rats indicated that the administration of IL-1β converting enzyme inhibitor caused a reduction of infarction size[40]. Minami et al. observed IL-lβ mRNA expression in the forebrain of the ischemic rat model as soon as 15 minutes which peaks in the cortex, hippocampus, striatum, and thalamus at 30 min and 240 min [41]. Buttini etal observed that the IL-lβ mRNA expression was at 15 minutes, peaked at 3 hours and vanished within 4 hours in spontaneous hypertensive rat's ischemic brain using in situ hybridization technology [42]. The results of our study about IL-1β were consistent with Chu.et al. who established that mRNA of IL-1β, in addition to more important pro -inflammatory cytokines, TNF-α and IL-6 were highly increased in the rat hippocampus after transient global cerebral I/R model through activation of P2X7 receptors [43]. In our study, we examined the anti-inflammatory effect of Empagliflozin in two treatment groups using two doses and we found a lowering in IL-1β level when I/R was performed in Empagliflozin pretreated groups. A recent study by Abdelhamid et al revealed that Empagliflozin ameliorated serum level of pro-inflammatory cytokine IL-1β in addition to IL-6 and TNF-α through down regulation NF-kB providing Hepatoprotective effect in alcoholic liver disease by mean of its anti-inflammatory properties [44], this showed consistency with the present study. Intercellular adhesion molecules (ICAM)-1 are upregulated on brain vascular endothelial cells following ischemic stroke and are considered crucial in Neutrophil recruiting as ICAM-1-deficient mice were observed recently safe from an experimental stroke [45]. Accumulation of Neutrophils in the brain is a characteristic feature of cerebral ischemia and is

considered key in worsening tissue injury [46]. Inflammatory cytokine IL-1β has been shown to boost the expression of ICAM-1 immediately after ischemia. In the ischemic brain, ICAM-1 rises within hours of the onset of stroke and peaks at around 12-48 hours [47]. Data from our work demonstrated an elevation in cerebral ICAM-1 level after 30 minutes of global ischemia followed by 60 minutes of reperfusion. Our study findings are in agreement with Li etal, this study established that transient MCAO caused a significant increase in ICAM-1 level in the ischemic brain[48]. As consistency with our research, Li and Liu explored the impact of ICAM-1 on the progression of the inflammatory response during I/R and its role in increasing cerebral damage, they show that ICAM-1 expression was elevated after induction of GCI in rat model reaching the peak level after 24 hours then began to reduce [49]. At the end of our work, the immunohistochemical detection of ICAM-1 revealed that Empagliflozin induced a significant reduction in ICAM-1 level as compared to control –vehicle group animals. Ojima et al demonstrated that Empagliflozin blocked the increase in ICAM-1 in the kidney of induced diabetic rats indicating the anti-inflammatory role of Empagliflozin in ameliorating diabetic nephropathy in experimental animals [50], in line with our findings, data from Steven etal study and Zhou et al study showed that Empagliflozin normalized the serum ICAM-1 in animal models of T2DM , as inflammation that induced in response to diabetes forms one of the underlying mechanisms of cardiovascular and other diabetic-related complications, the study demonstrated the role of Empagliflozin in maintaining the vascular integrity through reduction of inflammation beside its glucose-lowering effect[51-52]. Oxidative stress (OS) has been implicated in the development of I/R-induced brain damage, where the inventory of ROS is produced by mitochondrial dysfunction, infiltrated Neutrophils, and activated microglia [53].F2-isoP (8-iso-PGF-2α) which is a product of non-cyclooxygenases but ROS mediated membrane phospholipid and lipoprotein peroxidation, is measured in blood or cerebrospinal fluid (CSF) as a useful predictor of oxidative stress in cerebral ischemia/reperfusion[54]. High levels of free radicals- induced lipid peroxidation products were associated with higher infarction volumes and more severe neurological dysfunction in laboratory models [55]. Our study showed an elevation in the brain tissue level of F2-isoprostane (8-iso-PGF2α) after global cerebral I/R.This finding was also indicated by Ge etal who found that F2- isoprostane increased significantly in a rat model after 10 min. of transient global cerebral I/R with different reperfusion times[56],in the same way, a clinical study by Yu etal found elevated plasma levels of 8-iso-PGF-2α after traumatic brain injury[57]. Besides, plasma 8-iso-PGF-2α levels of patients were strongly associated with disease severity. Thus, the 8-iso-PGF2α can indicate the degree of functional impairments [57]. On a related note, Lorezano et al established that high levels of hyper acute plasma F2-isoprostane independently anticipate the development of infarct growth and infarct growth volume in acute ischemic stroke patients, indicating that oxidative stress can promote brain tissue damage and cell death[14]. At the end of our study, we found that the cerebral level of F2-isoprostane(8-iso-prostaglandin F2α) was significantly lower in both Empagliflozin pre-treated groups(5mg/kg and 10mg/kg) as compared to the control vehicle group, whoever there is no significant difference in the F2-isoprostane level between the two Empagliflozin groups. In coherence with our findings, in rat models of type 1 diabetes, Empagliflozin lowers several markers of oxidative stress including 8-iso-prostaglandin [58]. In addition, Empagliflozin has been documented to lower 8-iso prostaglandin F2α (8-iso-PGF2) as a marker for lipid peroxidation in patients with T2D, as a substantial drop was measured in fasting urinary 8-iso-PGF2α 28 days following treatment with Empagliflozin 10 mg/day (-45.5%) and Empagliflozin 25 mg/day (approximately -50.5%) compared to placebo was found in the a randomized study byNishimura etal [59].

5. Conclusion:

This study verified the anti-inflammatory and anti-oxidative effect of Empagliflozin as it ameliorates cerebral I/R injury by reducing the level of the pro-inflammatory and oxidative stress markers.

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