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Protective Effect of Ipragliflozin on Acute Brain Injury Induced by Endotoxemia in Mice

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ABSTRACT

Aims This study was done to investigate the potential neuroprotective effect of ipragliflozin during endotoxemia in mice.

Materials & Methods Twenty-four adult male Swiss-albino mice aged 8-12 weeks (25-35g) were randomized into four equal groups (n=6): sham (laparotomy without cecal ligation and puncture (CLP), sepsis (laparotomy with CLP), vehicle (equivalent volume of DMSO before CLP), and ipragliflozin (3mg/kg/day, orally before CLP). Tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), IL-1β, toll-like receptor 4 (TLR4), and P-signal transducer and activator of transcription (STAT)-3 levels were assessed in the brain tissue and histological examination was done.

Findings The tissue levels of TNF-α, IL-6, and IL-1B in the sham group were much lower than in the sepsis and vehicle groups. Furthermore, the ipragliflozin group had considerably lower tissue levels of TNF-α, IL-6, and IL-1B compared to the sepsis and vehicle groups. However, the sham group showed much lower tissue levels of TLR4 and STAT3 compared to the sepsis and vehicle groups. Also, the tissue levels of TLR4 and STAT3 in the ipragliflozin group were considerably lower than those in the sepsis and vehicle groups. Histopathology analysis demonstrated that ipragliflozin might considerably reduce brain damage compared to sepsis and vehicle groups that showed interstitial edema and included glial cells with pyknotic nuclei.

Conclusion Ipragliflozin attenuates brain dysfunction during CLP-induced polymicrobial sepsis in male mice.

Keywords Endotoxemia; Sepsis; Tumor necrosis factor-alpha; Toll-like receptor 4

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Introduction

Endotoxemia basically refers to the presence of endotoxins in blood derived from Gram-negative rod-shaped bacteria, causing hemorrhages, necrosis of the major tissues, and shock. Surgical procedures, chronic infection, and aging can all contribute to the rise of circulating endotoxin levels and endotoxemia [1]. The cecal ligation and puncture (CLP) model induces lipopolysaccharide (LPS), which is the major component of Gram-negative bacteria, and has been studied as a crucial modulator of the pathogenesis of bacterial infection and plays a crucial role in endotoxic shock [2].

Endotoxin acts as a potent stimulus for the release of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), and IL-6 that have been implicated in the pathophysiology of severe infectious diseases [3].

Sepsis is an infection-related systemic inflammatory response that in severe situations, can lead to septic shock and multiple organ failure syndromes [4]. In addition to neurotransmitter alterations, inflammatory cytokines, oxidative damage, mitochondrial dysfunction, apoptosis, and other variables, sepsis-associated encephalopathy (SAE), which develops as a result of diffuse encephalopathy brought on by an infection, also has a multifactorial pathophysiology [5]; however, the precise mechanisms involved are still unknown.

Inflammatory diseases have been demonstrated to be caused by abnormalities in cytokine receptor signaling pathways [6]. Hence, signal transduction pathways implicated in cytokine synthesis may also be crucial in sepsis. Bacterial endotoxins can trigger signal transducer and activator of transcription (STAT)-3 tyrosine phosphorylation [7]. NF-κB translocation is induced by activated STAT3, which then starts the production of cytokines [8].

Toll-like receptors (TLRs) are type I transmembrane proteins and are a panel of conserved pattern-recognition receptors (PRR) that are activated by a variety of pathogen-associated molecular patterns (PAMPs), thus initiating an innate immune response and inflammation in higher animals [9, 10].

TLR4 is confirmed to recognize LPS and regulates the production of proinflammatory cytokines, such as TNF-α and IL-1β [11], and mediates infiltration and activation of inflammatory cells to respond to infectious pathogens [12].

Ipragliflozin, a selective sodium-glucose (SGLT2) inhibitor developed in collaboration with Astellas Pharma and Kotobuki, was the first drug in its class to be approved for type 2 diabetes in Japan in 2014 [13]. Ipragliflozin is typically taken at a dose of 50 mg per day, while a dose of 100 mg per day is permitted in certain circumstances (insufficient efficacy). Sodium-glucose co-transporters (SGLTs) are a group of proteins that are found on the cell membranes of different tissues [14]. The transport of glucose in the small intestine and kidney is significantly influenced by two SGLTs, particularly, SGLT1 and SGLT2. The renal proximal tubules' S3 segment is where the high-affinity, low-capacity Na+/glucose cotransporter SGLT1 is located. It is widely distributed in the small intestine [15]. As opposed to this, SGLT2, a low-affinity, high-capacity Na+/glucose cotransporter, is mostly found in the S1 and S2 segments of the renal proximal tubules and is essential for the reabsorption of filtered glucose through the glomeruli [15-17]. Inhibition of SGLT2 is a unique strategy for the treatment of patients with type 2 diabetes mellitus (T2DM) since increased expression of SGLT2 in T2DM enhances the maximum reabsorption/transport capacity for glucose [18].

Ipragliflozin lowers blood sugar levels by preventing SGLT2-mediated glucose reabsorption in the renal proximal tubule and increasing glucose excretion in the urine [19, 20]. In large-scale postmarketing surveillance studies in the actual clinical setting in Japan, as well as in preapproval clinical trials involving patients with T2DM (including old patients), ipragliflozin's safety and effectiveness have been established [21-24]. In addition, a multicenter, randomized, placebo-controlled, double-blind research in insulin-treated individuals with T2DM demonstrated that ipragliflozin was both well-tolerated and efficient [25]. We investigated the neuroprotective effects of ipragliflozin through the modulation of proinflammatory mediators during endotoxemia.

Materials and Methods

Animals

Twenty-four adult male albino Swiss mice (25-35g) gained from the animal house of the College of Science in Baghdad University were kept at a constant temperature of 25°C and humidity of 60-65%, under a 12 h light/12 h dark in the animal house of Faculty of Sciences, University of Kufa.

Research design

This study was done at the Pharmacology and Therapeutics Department and Middle Euphrates Unit for Cancer Researches, Faculty of Medicine, University of Kufa. The mice were divided randomly into four groups (six mice per group): Sham group: Mice were given anesthesia before a laparotomy surgery but without CLP.

CLP-operated group (sepsis group): Mice underwent CLP.

Vehicle group: Mice were given an equal volume of dimethyl sulfoxide (DMSO) (Sigma Aldrich, Germany) orally once a day for seven days before CLP.

Ipraglflozin-pretreated group: Mice were treated with ipragliflozin orally once a day for seven days before CLP (pure powder was used, obtained from MedChem Express, USA).

Ethical considerations

The research was approved by the Bioethics Committee of the University of Kufa and its representative in the Faculty of Medicine (No. 7925; 30/3/2022). All procedures were done according to the recommendations of this committee.

Induction of sepsis

The Polymicrobial sepsis in mice was induced by CLP. After anesthesia of mice by an intraperitoneal injection of ketamine (100mg/kg, Alfasan Woerden, Holland) and xylazine (10mg, Alfasan Woerden, Holland), abdominal laparotomy was done (1.5cm midline incision) to expose the cecum. Then, it was ligated below the ileocecal valve and punctured twice with a G-20 needle before being returned to its anatomical location, the abdomen was sutured with a 5.0 surgical suture. Every 4h, mice were checked for various indicators of illness for 24h [26, 27].

Collection of samples

Twenty-four hours after CLP, mice were anesthetized and then beheaded; brains were separated and rinsed in ice-cold phosphate buffer solution, sectioned into two major coronal slices, one of which was retained in 10% formalin (Fluka Company, Switzerland) for histological investigation and the other part homogenized for the measurements of inflammatory markers. The final slice was mixed in a 1:10 (w/v) ratio with ice-cold 0.01 M (PBS; PH 7.4) with 0.5% cocktail protease inhibitor (Medchem Express, USA), and then processed with an ultrasonic liquid processor (Sonics, USA). The homogenates were then centrifuged at 15,000g for 30min at 4°C, and the supernatants were removed and kept at -80°C for the detection of additional markers using the ELISA method [28].

Measurements of brain inflammatory mediators (TNF-α, IL1β, IL-6, TLR4, and STAT3)

The brain tissues were homogenized first by combining in a 1:10 (w/v) ratio with ice-cold 0.01M (PBS; PH 7.4) with 0.5% cocktail protease inhibitor (Medchem Express, USA), and then processed with an ultrasonic liquid processor (Sonics, USA). The homogenates were then centrifuged at 15,000g for 30min at 4°C, and the supernatants were removed and kept at -80°C for the detection of markers using the ELISA method [28].

All Elisa kits for the measurements of brain inflammatory mediators were obtained from Bioassay Technology Laboratory, China, and used according to kit instructions.

The ELISA technique was done as follows: a plate with 96 wells was pre-coated with mouse antibodies. The inflammatory mediator present in the sample was added and bound to antibodies coated on the wells, then a biotinylated mouse antibody was added to bind to the inflammatory mediator in the sample. Then streptavidin-HRP was added and bound to a mouse biotinylated antibody. After incubation, unbound streptavidin-HRP was washed away during a washing step. Then, the substrate solution was added to the wells, which caused blue color development.

The intensity of the color depended on the quantity of the bound inflammatory mediator. The reaction was terminated by the addition of an acidic stop solution, which caused a color change from blue to yellow, and the absorbance was measured at 450nm by an Elisa reader (Bio/Tek ELx800, USA).

Histopathological examination

The formalin-fixed slices were tissue-processed to embed paraffin wax before being longitudinally cut into 5µm pieces. Hematoxylin and Eosin staining was used to stain the sections for histopathological examination [29] by a professional pathologist who was not aware of the study's design or the classification of animal groups.

The brain injury was scored [30]; zero for no morphological signs of damage, one for edema, eosinophilic dark (Pyknotic) neurons, or dark shrinking cerebral purkenje cells, two for at least two small hemorrhages, and three for clearly infarctive foci (local necrosis).

Statistical analysis

Statistical analysis was done using SPSS version 26. The normality of data was tested by the Kolmogorov-Smirnov and Shapiro-Wilk tests. The one-way ANOVA was applied for normally distributed data (parametric data) and the Chi-square test was used for the analysis of nonparametric data, at a significance level of 0.05.

Findings

Ipragliflozin effect on pro-inflammatory markers (TNF-α, IL-6, and IL-1β)

When comparing the sepsis and vehicle groups to the sham group, the brain levels of TNF-α, IL-6, and IL-1β were considerably higher in the sepsis and vehicle groups ($p<0.05$). The brain levels of TNF-α, IL-6, and IL-1β were lower in the ipragliflozin-treated group (Table 1).

Ipragliflozin down-regulated TLR4\STAT3 expression

The expression levels of TLR4\STAT3 in brain tissue in the sham group were significantly ($p<0.05$) lower than those in both sepsis and vehicle groups. On the other hand, the brain tissue levels of TLR4\STAT3 of the ipragliflozin-treated group were significantly ($p<0.05$) lower than those in both sepsis and vehicle groups (Table 1).

Effect of Ipragliflozin on brain histopathology

The ipragliflozin-pretreated mice showed mild architectural alterations compared to the sham group, which showed normal brain architecture. In terms of histopathological grading of normal brain tissue, this group showed mild degrees of damage (Figure 1).

Table 1) The brain tissue levels of inflammatory markers in the four experimental groups (n = 6 per group)

Inflammatory markers	Sham group	CLP group	Vehicle group (DMSO)	Ipragliflozin group	p-value
IL-6 (pg/ml)	183.11±4.3	219.27±6.3*	225.14±2.02**	188.4±3.3***	* 0.0001 ** 0.3 *** 0.0001
IL-1β (ng/L)	662.25±49.05	924.75±25.9*	920.52±16.57**	682.5±26.38***	* 0.0001 ** 0.9 *** 0.0001
TNF-α (ng/L)	176.61±3.02	241.56±13.7*	226.4±4.8**	182±6.4***	* 0.0001 ** 0.137 *** 0.0001
P-STAT3 (ng/ml)	34.6±1.5	72.8±3.2*	72.02±3.2**	56.5±0.7***	* 0.0001 ** 0.9 *** 0.006
TLR4 (ng/ml)	3.33±0.6	7.64±0.41867*	5.75±0.2**	4.07±0.3***	* 0.0001 ** 0.002 *** 0.0001

Data are expressed as mean ± standard error of the mean.
IL-6: interleukin-6; TNF-α: Tumor necrosis factor-alpha; P-STAT3: P-signal transducer and activator of transcription (STAT)-3; TLR4: toll-like receptor 4.
According to the one-way ANOVA: * CLP compared to the sham group; ** CLP compared to the vehicle group; ***CLP compared to the ipragliflozin group.

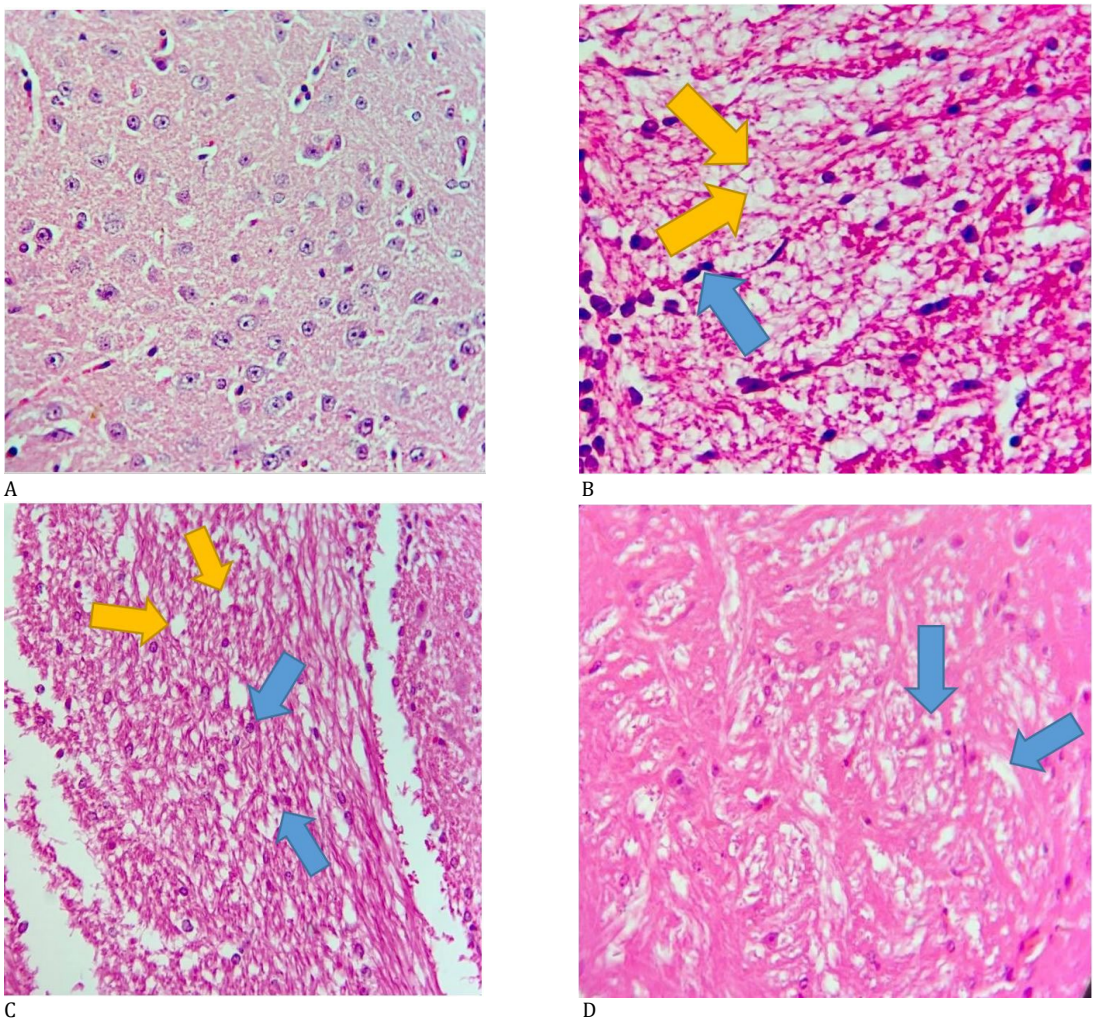

Figure 1) Histopathological examination of brain sections (x400). **A**: Sham group with normal histology and normal cerebrum. **B**: Sepsis group with a score of 1, showing changes, such as a cerebrum with interstitial edema (yellow arrows) and the presence of glial cells with pyknotic nuclei (blue arrows). **C**: vehicle group with a score of 1, showing changes, including a cerebrum with interstitial edema (yellow arrows) and the presence of glial cells with pyknotic nuclei (blue arrows). **D**: Ipragliflozin-pretreated group with a score of 0, showing changes, including a cerebrum with focal interstitial edema (blue arrows).

Discussion

Sepsis is a generalized inflammatory response that involves organ systems distant from the site of the initial infectious insult [31].

Many organ dysfunctions, particularly in the brain, make sepsis more complicated. Brain dysfunction can occur in people with no known etiological agents, suggesting that it is not linked to a direct central

nervous system (CNS) infection but rather to mediators generated during sepsis. This brain malfunction frequently manifests as an alteration in consciousness (known as encephalopathy), which can vary from a change in consciousness, often known as encephalopathy, ranging from confusion and delirium to coma. Otherwise, Focal neurological symptoms may be obvious and should prompt one to consider a localized brain lesion, often ischemic in nature [32]. The term "brain dysfunction" is used by the writers because "encephalopathy" is too limited. Recent evidence of long-term cognitive deterioration following sepsis supports its use**.** It is a common belief that encephalopathy can be reversed. Increased mortality and morbidity, and long-term cognitive impairment are all linked to sepsis-induced brain dysfunction (SIBD) [33]. In order to create specialized therapy strategies, an understanding of its pathophysiology is necessary. Neurological tests must be carried out every day to detect brain dysfunction [34], and this must direct the indications for additional research.

This experimental study reported a significant elevation in TNF-α, IL-6, and IL-1$_{\beta}$ in brain tissues in both control and vehicle groups in comparison with the sham group after sepsis. This finding is in agreement with other studies. Sepsis-induced CNS dysfunction is related to the local production of pro- and anti-inflammatory cytokines, altered cerebral microcirculation, neurotransmitter imbalance, apoptosis, and cognitive impairment. It is well established that IL-1β is one of the first cytokines to undergo modification, and after 24h of CLP, its levels are highly elevated [35]. CLP surgery dramatically increased the levels of IL-1β, IL-6, and TNF-α in plasma compared to the Sham group [36]. The levels of TNF-α, IL1β, and IL-6 were significantly elevated after 24h of CLP operation [37].

The results of this study showed that Ipragliflozin-treated groups had considerably lower brain tissue levels of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) than those in the sepsis and vehicle groups. This finding is in agreement with other studies. One experimental study showed that ipragliflozin reduced inflammatory markers (IL-6 and TNF-α) [38]. Furthermore, the treatment with ipragliflozin resulted in diminished levels of IL-6, IL-1β, and TNF-α [39, 40].

This experimental study reported a significant increase in the expression of TLR4 in brain tissues in both control and vehicle groups in comparison with the sham group after sepsis. This finding is in agreement with other studies. Mice's brain tissue subjected to CLP showed neuroinflammation and microglia activation (the major active immune cells in the CNS). When microglia were activated, an increase was found in TLR-4 mRNA or protein expression levels in the brain [41].

This experimental study reported a significant increase in the expression of P-STAT3 in brain tissues in both control and vehicle groups in comparison with the sham group after sepsis. This finding is in agreement with other studies. One experimental study reported that the levels of p-STAT3 increased in the CLP group [42].

The present study showed that Ipragliflozin-pretreated groups had significantly lower brain tissue levels of TLR4 compared to the sepsis and vehicles groups.

One experimental study indicated suppressed expression of TLR4 after ipragliflozin pre-treatment [43]. The present study showed that Ipragliflozin-pretreated groups had significantly lower brain tissue levels of STAT-3 compared to the sepsis and vehicles groups. Our finding is in agreement with other experimental studies. One experimental study showed that ipragliflozin attenuated the up-activity and activation of the STAT3 pathway [44].

The present study showed that the sepsis and vehicle groups had significantly higher degrees of brain tissue injury compared to the sham group.

Histopathological findings in the sepsis and vehicle groups showed the cerebrum with interstitial edema, the presence of glial cells with pyknotic nuclei, and the activation of astrocytes and microglia (increased cellularity). This finding is in agreement with other experimental studies. Altaş *et al.* showed in shrunken neurons, eosinophilic cell bodies, disruption of certain microvessels, meningeal congestion, bleeding, edema, and pyknotic nuclei the ischemia-reperfusion injury (I/R) group [45].

Brain tissues obtained from mice treated with ipragliflozin showed less cellular damage at the same time. This result indicated that ipragliflozin could protect against sepsis and prevent brain dysfunction. This finding is in agreement with other experimental studies. SGLT-2 as an SGLT2 inhibitor, caused the progression of diabetic microvascular complications, such as neuropathy [46].

Conclusion

Ipragliflozin can attenuate brain injury during CLP-induced polymicrobial sepsis in male mice.

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Ethical Permission: The research was approved by the Bioethics Committee of the University of Kufa and its representative in the Faculty of Medicine (No. 7925; 30/3/2022). All procedures were done according to the recommendations of this committee.

Conflicts of Interests: All authors declare that there is no conflict of interest in the study.

Authors' Contributions: Mohammad AR (First Author), Introduction Writer/Discussion Writer /Main Researcher (40%); Shnaien AA (Second author) Assistant Researcher/Statistical Analyst (30%), Alabsawy SK (Third Author), Assistant Researcher/Results Writer (20%), Hassan ES (Fourth author) Assistant Researcher/Methods Writer (10%)

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