Original Article

Effects of Empagliflozin on a Model of Chronic Depression and Brain Toll-Like Receptors Gene Expression in Male Rats

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Abstract

Background: Empagliflozin is an antidiabetic medication having anti-inflammatory and antioxidant properties. **Objectives:** To develop a chronic unpredictable stress (CUS) model in male rats, evaluate empagliflozin's antidepressant effects, and describe the link between stress, an antioxidant enzyme, and Toll-like receptor (TLR)-4 gene expression in male rats. **Materials and Methods:** In this experiment, 50 rats were divided into five groups: G1–G5. The forced swimming test (FST) was used to examine the antidepressant effects of fluoxetine and empagliflozin, and real-time polymerase chain reaction was used to measure TLR-4 gene expression. FST was performed on each rat on days 0, 10, and 25. CUS was performed on each rat for 24 days. **Results:** By the end of day 10, all animals subjected to the CUS program had a substantial ($P < 0.05$) increase in immobility duration compared with day 0. The immobility duration in the CUS group was statistically significantly greater than the baseline. Stressed rats demonstrated a statistically significant reduction in immobility duration compared with CUS group G2. In comparison to the CUS group, fluoxetine significantly reduced immobility duration ($P < 0.05$). In terms of gene expression, the mean of fold changes in TLR-4 mRNA level in group 2 was considerably greater than in group 1. When compared with group 2, the means of the fold changes in TLR-4 mRNA level were considerably lower in groups 3, 4, and 5. **Conclusions:** Empagliflozin has antidepressant-like effects and can counteract the impact of stress-induced TLR-4 overexpression in the hippocampus and elevate the activity of antioxidant enzymes in the brains of depressed rats.

Keywords: Antioxidant, chronic unpredictable stress, depression, empagliflozin, TLR-4

Introduction

Depression is a worldwide mental condition caused by a variety of factors.[1] Its major clinical signs are often a gloomy mood, lack of desire, loss of interest, loss of pleasure, and cognitive impairment.[2] Although several medications are already accessible for depression treatment, they frequently cause numerous major clinical issues, such as adverse reactions.[3] At some point in their lives, one in every five women and one in every eight men will have a severe depressive episode.[4] While depression is a complicated condition with several underlying causes, the specific mechanism through which it emerges is yet unknown. Hereditary, epigenetic, and environmental variables seem to account for significant variances in the hypothalamic-hypophyseal-adrenal axis and low levels of monoamine neurotransmitters.[5]

Lower dopamine (DA) levels in those suffering from serious depression and decreased cerebrospinal fluid monoamine metabolite levels in people who tried suicide and had depression show the significant role of monoamine levels in the depression process.[6] Also, in depressed individuals, stimulating the serotonergic pathway can enhance DA release.[7]

As a consequence, the serotonergic and dopaminergic systems may interact. Modulation of Toll-like receptors (TLRs) is another important mechanism that may shed light on the pathophysiology of depression.[8]

TLRs are a class of pattern recognition receptors that recognize microbes' pathogen-associated molecular patterns

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(PAMPs) and damage-associated molecular patterns (DAMPs). The activation of these receptors by their respective PAMPs or DAMPs may result in the production of proinflammatory cytokines as well as the generation of innate and acquired immune responses.[9]

Stress causes the production of DAMPs, which bind to TLRs and produce inflammation, which plays an important part in the etiology of depression.[10] Tricyclic antidepressants, monoamine oxidase inhibitors, selective serotonin reuptake inhibitors, serotonin-norepinephrine reuptake inhibitors, and norepinephrine and dopamine reuptake inhibitors are antidepressants used to treat depression. Empagliflozin is an antidiabetic medication that works by blocking insulin-independent renal glucose reabsorption in the proximal tubules.[11] Empagliflozin (EMP) is a chemical having anti-inflammatory and antioxidant properties.[12] As a result, the current investigation aims to evaluate the influence of empagliflozin on a model of chronic unpredictable stress (CUS), TLR-4 gene expression, and antioxidant enzymes in the brains of male rats.

Materials and Methods

Animals

In this experiment, 50 male adult albino rats were employed. Their weights ranged between 245 and 300 g. The rats were housed in the animal house of the College of Medicine/ University of Babylon and were maintained at 25° 14 h of light and 10 h of darkness, with water and food. The animals were randomly separated into groups after 2 weeks of adaption, according to the experimental procedure.

General experimental procedure

1. Forced swimming test (FST) was performed on each rat on days 0, 10, and, 25.
2. The rats in group 1, not exposed to chronic unpredictable stress and not treated with any medication.
3. CUS was conducted on each rat in groups 2 (positive control), 3, 4, and 5 for 24 days.
4. Without treatment, each rat in group 2 received 0.5 mL of distilled water.
5. For 2 weeks, each rat in group 3 was given 10 mg/kg/day P.O.[13]
6. For 2 weeks, each rat in groups 4 and 5 were given 10 and 20 mg/kg/day, respectively.

Chronic unpredictable stress

The CUS was conducted on each rat in groups 2, 3, 4, and 5 for 24 days. The stressors that used were 15 min forced swimming, 12 h crowded cage, 12 h cage tilting (45°), 1 h restraint, reversal of the light/dark cycle, 12 h wet bedding, 24 h food deprivation, 24 h food and water deprivation, Tail pinch, and 12 h water deprivation. Each day, the rats were exposed to two different types of stressors to prevent their adaptation to these stressors.[14]

Forced swimming test

This behavioral test is frequently employed to gauge "despair" behavior in animal models of depression.[15]

In the FST, rats were subjected to an unavoidable swimming condition, in which they had to swim in a water-filled cylindrical container until they stopped struggling after a time of vigorous exertion. As previously conducted,[16] rats were taught to swim for 15 min before the test, which was repeated the next day under identical conditions but with a 5-min time limit. Following each animal test, water was refreshed and the cylinder was thoroughly cleaned. To prevent hypothermia, the animals were then dried and heated.[17]

Following the recording of the test, the duration of each animal's total immobility was determined.

Tissues samples preparations

The animals were beheaded on the 25th day, 24 h after their final treatment. After the incision of the skull from the posterior foramen magnum, the brains were extracted. The cerebellum and olfactory bulbs were removed. The hippocampus, which has a banana-like form, was isolated from the rest of the brain using anatomical tools. The isolated hippocampus was directly kept in Trizol reagent and transferred to the laboratory for RNA extraction.

Primers

The NCBI-Gene Bank database and the online Primer 3 design program were used to create the primers for the target gene (TRL-4) and the housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) [Table 1].

RNA extraction and cDNAs synthesis

Total RNA Extraction Kit AccuZolTM (Bioneer, Daejeon, South Korea) was used to get the total RNA from hippocampus tissue, and the procedure was carried out according to manufacturer. Then, the Nanodrop

Table 1: Primers of TLR-4 and GAPDH

Primer		Sequence (5'-3')	Product size	NCBI reference
TLR-4 gene	F	TCCGCTGGTTGCAGAAAATG	84 bp	NM_019178.2
	R	AGGCAGGAAAGGAACAATGC		
GAPDH gene	F	AGTTCAACGGCACAGTCAAG	99 bp	NM_017008.4
	R	CCCATTTGATGTTAGCGGGATC		

spectrophotometer (THERMO, Waltham, United States) was used to measure and assess the extracted total RNA. After that, DNase I enzyme kit (Promega Company, Madison, United States) was used to eliminate the minute quantities of DNA from the isolated total RNA. After that, DNase I enzyme kit (Promega Company) was used to eliminate the minute quantities of DNA from the isolated total RNA. Finally, The TLR-4 and GAPDH genes' cDNAs were produced by using the M-MLV Reverse Transcriptase kit as per the manufacturer's instructions.

Quantitative polymerase chain reaction

The SYBR green reporter dye was used to evaluate relative RNA expression in a real-time quantitative polymerase chain reaction (PCR).

Using Bio-Rad iQ5 detection equipment (Bio-Rad, Richmond, Virginia), template cDNAs from each sample were tested for quantitative expression levels of the TLR-4 gene and a housekeeping gene GAPDH.

Glyceraldehyde-3-phosphate dehydrogenase

It is a critical regulatory enzyme that catalyzes the oxidative phosphorylation of glyceraldehyde-3-phosphate during glycolysis and is one of the most often utilized housekeeping genes in gene research. Housekeeping genes, including GAPDH, are frequently utilized as reference genes because their expression levels stay reasonably steady in response to any treatment.[18]

Antioxidant enzyme measurement

Superoxide dismutase and catalase were measured according to superoxide dismutase (SOD) kit (SolarBio, Beijing, China) and Catalase kit (SolarBio, Beijing, China) protocols, and the optical density was measured for each sample using a spectrophotometer (CYAN, Hulshout, Belgium), then determine the activity of each enzyme.

Statistics analysis

The SPSS program version 25 (SPSS, IBM Company, Chicago, Illinois) for Windows 10 was utilized. The data were given as a mean ± standard error of the mean (SEM). The post hoc test and one-way analysis of variance (Turkey) were used in the statistical analysis. A *P*-value of less than 0.05 was considered statistically significant.

Results

Forced swimming test

Comparison of mean immobility time among the rat groups

There were no significant differences ($P < 0.05$) in the mean of immobility period on days 10 and 25 in comparison to day 0 in a control group that was not administered any drug and unexposed to CUS, whereas in an animal that was exposed to CUS only (G2), the mean immobility period on days 10 and 25 significantly elevated ($P < 0.05$) compared with day 0 [Table 2].

Furthermore, the mean immobility time greatly rised on day 10 ($P < 0.05$) in contrast to day 0 in groups 3 (administered 10 mg/kg fluoxetine for 2 weeks days + CUS), 4 (administered 10 mg/kg EMP + CUS), and 5 (administered 20 mg/kg EMP + CUS).

On day 25, there was a significant decrease in immobility time compared with (day 10) in G3, G4, and G5.

Comparison of the mean of immobility time among rat groups on day 25

On day 25, there was a significant ($P < 0.05$) increase in the mean of immobility time in G2 compared with G1, and a significant decrease in the mean of immobility time in G3, G4, and G5 compared with G2 [Figure 1].

Gene expression results

There was a significant increase ($P < 0.05$) in the mean of TLR-4 gene fold changes in rats that were exposed to CUS for 24 days (G2) compared with rats in the normal group (G1). In rats being under CUS and treated with 10 mg/kg for 14 days (G3), a significant ($P < 0.05$) decrease in the mean of gene expression of TLR-4 [Figure 2].

Rats that were exposed to CUS and given EMP (10 mg/kg) for 14 days periods (G4) show a significant ($P < 0.05$) decrease in the mean of TLR-4 gene fold changes. In group 5, in which rats were exposed to CUS and treated with 20 mg/kg for 14 days, a very significant ($P < 0.05$) decreased in the mean of TLR-4 gene fold changes. Also, there was a significant decrease ($P < 0.05$) in the mean of TLR-4 gene fold in G5 compared with G3 [Figure 2].

G2 (untreated and exposed to CUS) showed fewer cycles that reflex high amount of TLR-4 gene. G3 (treated with 10 mg /kg fluoxetine+ CUS for 14 days), G4 (treated with

Table 2: Comparison of the mean ± SEM immobility time among rat groups on days 0, 10, and 25

FST	G1	G2	G3	G4	G5
Day 0	42.7 ± 1.11	43 ± 1.95	41.5 ± 1.66	46.2 ± 1.55	39.7 ± 1.23
Day 10	44.6 ± 1.88	70.3 ± 1.83[a]	70 ± 1.93[a]	71.9 ± 1.21[a]	66.3 ± 1.86[a]
Day 25	47 ± 1.27	73.8 ± 2.11[a]	46.6 ± 1.57[b]	49.2 ± 0.84[b]	43.8 ± 1.31[b]

FST: forced swimming test, G1: normal control, G2: positive control, G3: treated with 10 mg/kg fluoxetine+ CUS for 14 days, G4: treated with 10 mg/kg EMP+ CUS, G5: treated with 20 mg/kg EMP+ CUS.

[a]Increased significantly compared with day 0.

[b]Decreased significantly compared with day 10

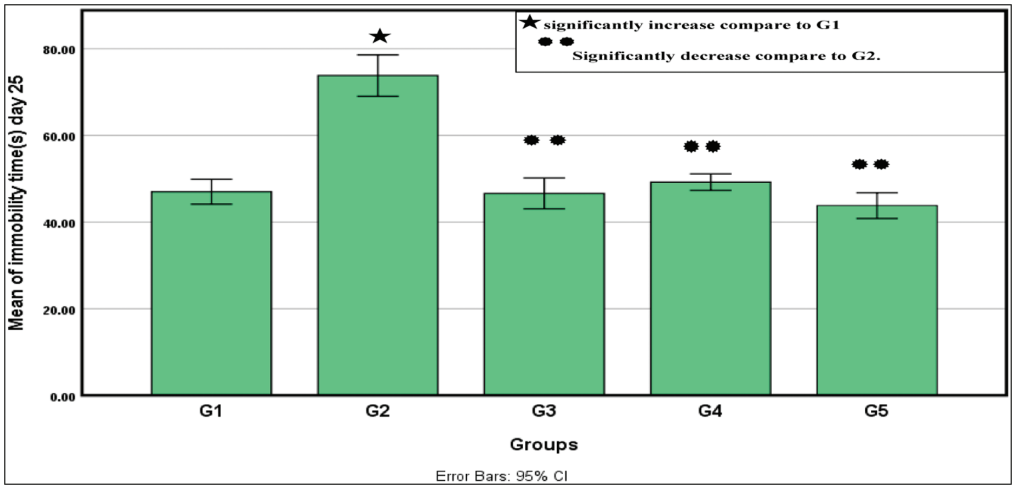

Figure 1: Comparison of the mean ± SEM of immobility time among rat groups on day 25. G1: normal control, G2: positive control, G3: treated with 10 mg/kg fluoxetine + CUS for 14 days, G4: treated with 10 mg/kg EMP + CUS, G5: treated with 20 mg/kg EMP + CUS

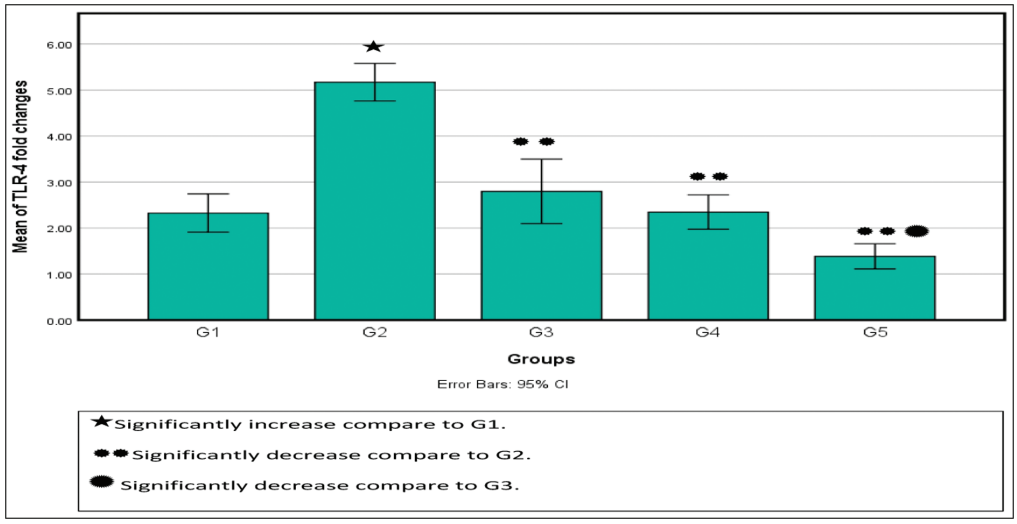

Figure 2: Comparison of TLR-4 fold changes mean ± SEM among rats groups on day 25. G1: normal control, G2: positive control, G3: treated with 10 mg/kg fluoxetine + CUS for 14 days, G4: treated with 10 mg/kg EMP + CUS, and G5: treated with 20 mg/kg EMP + CUS

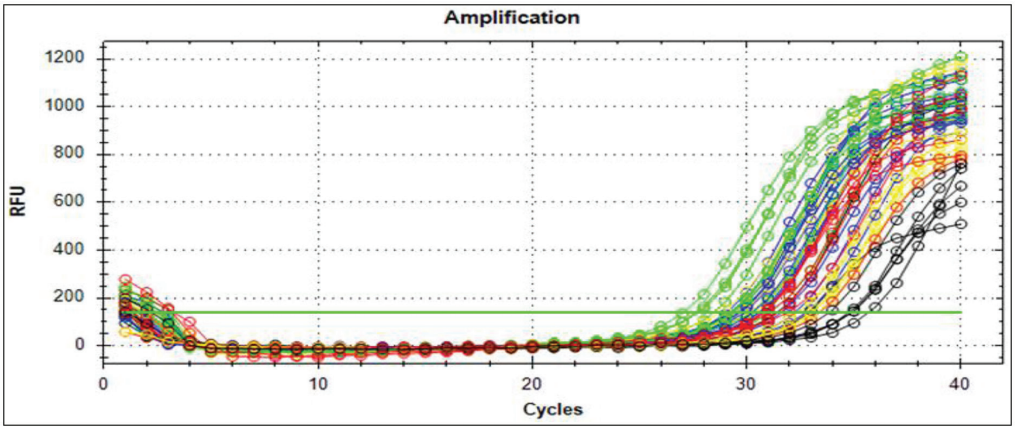

Figure 3: The real-time PCR amplification plots of TLT-4 gene expression in the hippocampal of rats. Yellow blot (G1: normal control), green blot (G2: positive control), blue blot (G3: treated with 10 mg/kg fluoxetine+ CUS for 14 days), red blot (G4: treated with 10 mg/kg EMP+ CUS), and black blot (G5: treated with 20 mg/kg EMP+ CUS)

10 mg/kg empagliflozin+ CUS), G5 (treated with 20 mg/kg empagliflozin+ CUS) showed a higher Ct value compared to G2. This indicates a low TLR-4 levels in these groups [Figure 3]. With regard to housekeeping gene, all groups showed the same Ct value of GAPDH amplification [Figure 4].

Antioxidant enzyme results

On day 25, there was a substantial (P = 0.05) drop in the mean SOD levels in rats subjected to CUS (G2) compared with animals in the control group (G1) [Table 3; Figure 5].

Similarly, when fluoxetine (10 mg/kg/day) (G3), EMP (10 mg/kg/day) (G4), and EMP (20 mg/kg/day) (G5) were compared with G2, the mean SOD concentration increased significantly [Table 3; Figure 5].

Additionally, on day 25, there was a significant decrease in the mean catalase concentration in G2 and G3 compared with G1, and a significant rise in the mean catalase concentration in G4 and G5 compared with G2 [Table 3; Figure 5]].

Discussion

Depression is a potentially fatal psychological illness that affects a large number of individuals worldwide. As a result, the goal of this study was to look at empagliflozin antidepressant effectiveness against CUS-induced depression utilizing FST, as well as its effect on TLR-4 gene expression.

To our knowledge, this is the first study to look at the effects of empagliflozin on chronic stress-induced TLR-4 gene expression in the hippocampus in male rats in addition to proinflammatory cytokines and antioxidant enzymes. In this study, it was observed that there were no statistically significant variations in immobility duration among rats from all groups on day 0.

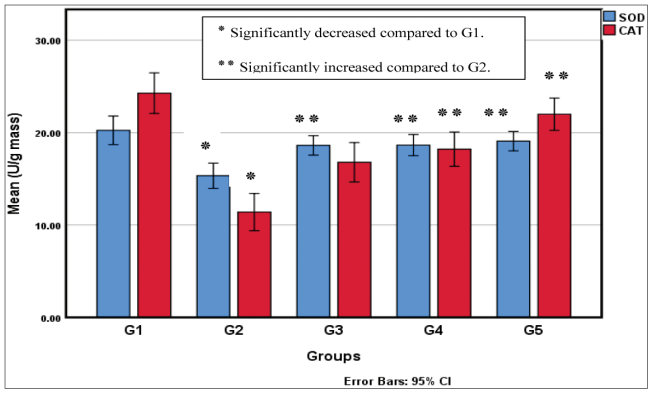

Figure 5: Comparison of superoxide dismutase and catalase mean ± SEM among rats groups on day 25. G1: normal control, G2: positive control, G3: treated with 10 mg/kg fluoxetine+ CUS for 14 days, G4: treated with 10 mg/kg EMP+ CUS, and G5: treated with 20 mg/kg EMP+ CUS

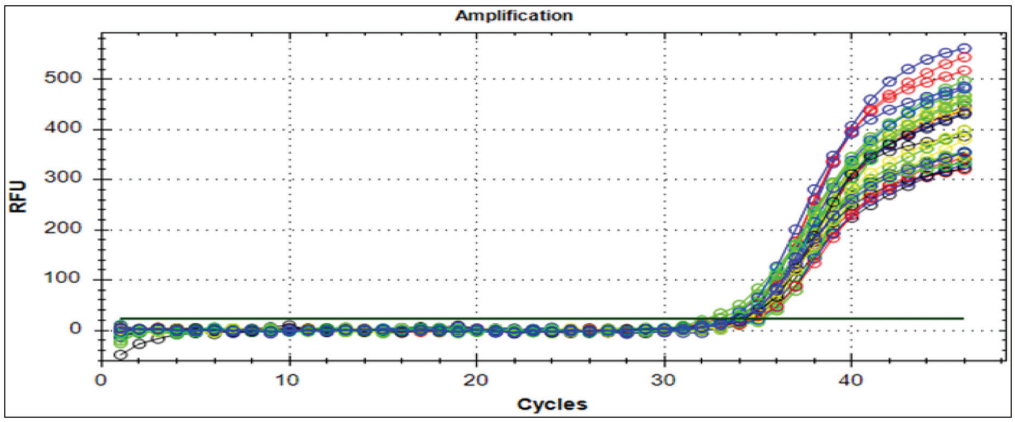

Figure 4: The real-time PCR amplification plots of housekeeping GAPDH gene expression hippocampal of rats. Yellow blot (G1: normal control), blot (G2: positive control), red blot (G3: treated with 10 mg/kg fluoxetine+ CUS for 14 days), green blot (G4: treated with 10 mg/kg EMP+ CUS), and black blot (G5: treated with 20 mg/kg EMP+ CUS)

Table 3: Comparison of SOD and CAT mean ± SEM among rats groups on day 25

Groups	G1	G2	G3	G4	G5
SOD	20.24 ± 0.68	14.26 ± 0.79[a]	18.94 ± 0.79[b]	17.51 ± 0.55[b]	17.84 ± 0.76[b]
CAT	24.27 ± 0.97	11.40 ± 0.89[a]	16.79 ± 0.94[b]	18.20 ± 0.81[b]	21.99 ± 0.77[b]

CAT: Catalase, G1: normal control, G2: positive control, G3: treated with 10 mg/kg fluoxetine+ CUS for 14 days, G4: treated with 10 mg/kg EMP+ CUS, G5: treated with 20 mg/kg EMP+ CUS, SOD: superoxide dismutase.

[a]Significantly decreased compared with G1.

[b]Significantly increased compared with G2

However, during 10 days of unpredictable stress, there was a significant increase in immobility period during FST. This suggests that these animals have developed a depression model.

Earlier research found that by subjecting rats to unexpected stress protocols utilizing various stressors, they could develop depression characteristics.[17,18] On day 25, group 2 (positive control) had depressive-like behavior, as evidenced by a substantial increase in immobility duration during FST compared with group 1 (normal control). These findings agreed with those of prior research.[18] When compared with rats exposed only to stress, the results of 14 days of treatment with empagliflozin 20 and 10 mg/kg with the continuation of chronic stress revealed a significant decrease in immobility time in these rats.

These findings indicated that fluoxetine and empagliflozin have an antidepressant effect, which was supported by another study.[19] Stress causes depressive symptoms by impairing serotonin neurotransmission, decreasing hippocampal neurogenesis, disrupting the hypothalamic-pituitary-adrenal axis, increasing oxidative stress, decreasing neuroplasticity and synaptic plasticity, and inducing inflammation in neurons via increased proinflammatory cytokine production.[20] The monoamine hypothesis is one of the various pathways suggested to lead to the development of depression.[21]

Indeed, this theory states that depression is connected to altered levels of the monoamine neurotransmitters such as serotonin (5-norepinephrine and dopamine reuptake inhibitors hydroxy tryptamine), noradrenaline, and DA. Fluoxetine acts by inhibiting the reuptake of serotonin, increasing serotonin activity.[22] With regard to TLR-4, the rats that were exposed to chronic stress for a period of 24 days showed an increase in the gene expression of TLR-4 in hippocampal tissue compared with the rats in the control group. TLR-4 is found in many different types of cells, such as macrophages, microglia, astrocytes, and neurons, and it regulates the adrenal response to stress and inflammatory stimuli as well as the cerebral response to stress.[23] This outcome is in line with previous findings indicating that stress-exposed animals had greater levels of TLR-4 mRNA in their prefrontal cortex and hippocampus.[24] Activation of TLR-4 likely contributes to the pathophysiology of stress-evoked depression.[25] On the other side, a reduction in TLR-4 gene expression was shown in groups treated with EMP 10 and 20 mg/kg/day for 24 days. The ability of empagliflozin to inhibit the gene expression of TLR-4 was found in studies conducted on rats, where it was found that empagliflozin at a dose of 10 mg/kg/day reduces the gene expression of TLR-4 in kidney tissues and protects them from stress.[26] Inhibiting TLR-4 gene expression significantly reduced depression-like behavior and improved depression symptoms.[27]

In terms of antioxidant enzymes, when it comes to antioxidant enzymes, on day 25, superoxide dismutase and catalase enzyme activity in the stressed rat group were considerably lower than in the control rat group.

The reason is that prolonged stress raises the quantity of free radicals, and antioxidant enzymes are the first line of defense against oxidative stress; most of these enzymes are consumed and, therefore, their concentration falls, leading to oxidative stress. High reactive oxygen species production and depleted antioxidant defenses stimulate proinflammatory signaling, causing damage to critical macromolecules and cellular death.[28]

Because of its increased oxygen demand, higher lipid content, and lower antioxidant defense, the brain is more vulnerable to oxidative stress.

This outcome contrasts previous research findings. Superoxide dismutase and catalase levels are significantly higher in the rat groups treated with 10 mg/kg/day EMP+ (chronic unpredictable mild stress), G5 (treated with 20 mg/kg/day EMP+ [CUMS], and 10 mg/kg fluoxetine plus CUMs for 14 days) than in the CUS-only group.

Many investigations have revealed that EMP restored or raised antioxidant levels in a range of tissues.[29]

In summary, this study found that prolonged unpredictable stress causes rats to acquire depressive-like behavior and a high expression of TLR-4 mRNA levels in the hippocampus. Empagliflozin exhibits antidepressant-like activity as measured by FST, and empagliflozin treatments inhibit stress-induced TLR-4 mRNA expression elevation. Also, empagliflozin increases antioxidant enzyme activity in the brain of rats.

Ethical consideration

The study was carried out in accordance with the ethical standards that have their origin in the Helsinki Declaration. The study protocol was reviewed and approved by a local ethics committee according to the reference number 4-1 on July 6, 2022, to get this approval.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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