Research in Pharmaceutical Sciences, February 2024; 19(1): 64-72
Received: 02-12-2022
Peer Reviewed: 27-02-2023
Revised: 13-05-2023
Accepted: 31-12-2023
Published: 06-02-2024

School of Pharmacy & Pharmaceutical Sciences
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Original Article

PI3K/AKT and STAT3 pathways mediate the neuroprotective effect of dasatinib from acute cerebral injury in endotoxemic mice

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Abstract

Background and purpose: Sepsis induces brain dysfunction and there is still a requirement for an unemployed viable restorative approach. This study aimed to investigate the role of dasatinib in the modulation of proinflammatory mediators, attenuating neuroinflammatory response, and it's signaling pathway during endotoxemia.

Experimental approach: Twenty-four adult male Swiss-albino mice were randomized into four groups: sham (undergo laparotomy without cecal ligation and puncture, sepsis (laparotomy with cecal ligation and puncture), vehicle-dimethyl sulfoxide, dasatinib (20 mg/kg/day) intraperitoneally. Brain tissue used for assessment of interleukin (IL)-6, IL-1β, tumor necrosis factor-alpha (TNF-α), IL-10, Toll-like receptor 4 (TLR4), protein kinase B (AKT), phosphoinositide 3-kinases (PI3K), signal transducer and activator of transcription 3 (STAT3), and histopathological examination.

Findings/Results: Brain tissue levels of TNF-α, IL-6, and IL1 β were higher in the sepsis group than in the sham and vehicle groups. The dasatinib group had considerably lower tissue levels of these markers and significantly higher tissue values of IL-10 than the sepsis and vehicle groups. The sham group had much lower tissue values of TLR4, AKT, STAT3, and PI3k than in sepsis and vehicle groups. Furthermore, tissue levels of these markers in the dasatinib group were considerably lower than those in the sepsis and vehicle groups. Histopathology demonstrated that dasatinib might considerably reduce brain damage and the intensity of neuroinflammation when compared to sepsis and vehicle groups that showed extensive brain inflammation and damage.

Conclusion and implication: Dasatinib attenuated endotoxemia-induced acute brain damage in mice *via* modulating effects on TLR4, PI3K, AKT, and STAT3 downstream signaling pathways.

Keywords: CLP; Dasatinib; Endotoxemia; Sepsis.

INTRODUCTION

Endotoxemia is characterized by the presence of endotoxins in the circulation, which are generated by gram-negative rod-shaped bacteria and induce hemorrhages, brain necrosis, and shock. Endotoxic shock is the most severe type of sepsis. The etiology of it is unknown (1). The cecal ligation and puncture model (CLP) induces lipopolysaccharide (LPS), the main element of gram-negative bacteria, it has been investigated as a critical modulator of bacterial infection pathogenesis and plays a critical part in endotoxic shock (2).

Sepsis is defined as the systemic inflammatory response to infection produced by a variety of pathogenic bacteria. It is a severe public health issue that can result in considerable patient death as well as an increase in public health care costs (3). The disorder begins with a localized infection caused by microorganisms such as bacteria, which progresses to tissue invasion *via* the bloodstream, resulting in the development of sepsis and septic shock (4).

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Access this article online	
	Website: http://rps.mui.ac.ir
	DOI: 10.4103/1735-5362.394821

Sepsis is frequently associated with acute brain dysfunction. Its etiology is exceedingly convoluted, with both inflammatory and noninflammatory mechanisms responsible for considerable changes in sensitive brain areas. Severe microglial excitation, reduced brain perfusion, damaged blood-brain barrier, and neurotransmission impairment are all important occurrences (5). Other research suggests that tumor necrosis factor-alpha (TNF-α) signaling *via* TNF receptor 1 (TNFR1) causes sepsis-induced brain inflammation characterized by aquaporin 4 overexpression, astrocyte activation, edema, neutrophil infiltration, and apoptotic cell death (6).

Pattern recognition receptors are critical elements of the innate immune system. Toll-like receptors (TLRs) are pattern recognition receptors that play a crucial role in recognizing exogenous and endogenous ligands and triggering intracellular signaling pathways, culminating in proinflammatory cytokines production. TLR4 is a TLR family identified and triggered by LPS (7). TNF-α and interleukin-1 beta (IL-1β) are among the proinflammatory cytokines increased in the brain during sepsis (8). These intermediaries affect the expression of neurons' alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors and N-methyl-D-aspartate receptors resulting in brain damage (9).

Dasatinib is a tyrosine kinase inhibitor with immunomodulatory effects and the ability to pass the blood-brain barrier. It, an FDA-approved medication, is now used to treat imatinib-resistant chronic myeloid leukemia. Dasatinib was first authorized in the European Union and United States in 2006 (10). It alters the amounts of pro-and anti-inflammatory cytokines generated by LPS in microglial cells and primary astrocytes, reduces TLR4-protein kinase B (AKT) and/or TLR4-extracellular receptor kinase (ERK) signaling in microglial cells, modulates LPS-stimulated neuroinflammatory responses (11). Hence, we aimed to investigate the role of dasatinib in the modulation of proinflammatory mediators, attenuating neuroinflammatory response, and its signaling pathway during endotoxemia.

MATERIALS AND METHODS

The study was carried out at the Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Kufa in the period between the 5th of January 2022 and the 20th of July 2022. The research was approved by the Bioethics Committee at the University of Kufa and its representation in the Faculty of Medicine (Ethical Approval No. 20580 on 2/1/2022).

The study approaches

Twenty-four adult male albino Swiss mice, weighing 25-35 g, aged 6-8 weeks, were obtained from the Animal Resources Centre in the Faculty of Sciences, University of Kufa, which were kept at a humidity of 60-65% and constant temperature of 25 °C, with a 12/12-h light/dark cycle. Mice were randomly divided into four groups (6 each) as follows:

(1) Sham: anesthesia was given and laparotomy surgery was done without CLP.
(2) Sepsis: cecal ligation and puncture were done.
(3) Vehicle: received DMSO intraperitoneally (i.p) once daily for 3 days, the last dose was given 1 h before CLP.
(4) Dasatinib: received a (20 mg/kg/daily) dasatinib i.p injection once daily for 3 days, the last dose was 1 h before CLP.

Experimental procedure

Cecal ligation and puncture

Mice were sedated with 100 mg/kg ketamine and 10 mg xylazine i.p. The cecum was revealed after an abdominal laparotomy through a 1.5 cm midline incision, then ligated just below the ileocecal valve and poked twice with a G-20 needle before being restored to its anatomical place. After that, the abdomen was sutured with a 5.0 surgical suture. Mice were checked every 4 h for 24 h for various indicators of illness before being returned to their cages (12,13).

Preparation of dasatinib

Dasatinib powder was obtained from Med Chem Express (United States) and prepared in diluted DMSO, then given in a dose of

(20 mg/kg/daily) i.p once daily for 3 days, the last dose was given 1 h before CLP (11).

Tissue preparation for TNF-α, IL-1β, IL-6, and IL-10

Mice were beheaded 24 h after CLP; brains were separated and cleaned in an ice-cold phosphate-buffered solution. They were maintained on ice and weighed before being sectioned into two major coronal slices, one of which was retained in 10% formalin for histological investigation. The final slice was homogenized by the ultrasonic liquid processor after being combined in a 1:10 (w/v) ratio with ice-cold 0.01 M phosphate-buffered solution (pH 7.4) containing 0.5% cocktail protease inhibitor and 0.5 % Triton X100. The homogenates were centrifuged at 15,000 *g* for 30 min at 4 °C, the supernatants were removed and kept at -80 °C for further ELISA measurements of markers (14-15)

Tissue preparation for histopathology

The formalin-fixed tissue slices were processed to embed paraffin wax before being longitudinally cut into 5 mm pieces. Then, Hematoxylin and Eiosin stain were used to stain the sections for histopathological examination (16). A senior pathologist performed the histological examination and was blinded to the study design and the allocation of each animal. The degree of brain injury was calculated as follows (17): when there were no morphological signs of damage, the score (0) was designated which was considered normal; if there was edema, eosinophilic dark (pyknotic) neurons, or dark shrunk cerebral Purkinje cells, the score was (1) considered slight; if there were at least two small hemorrhages, the score was (2) considered moderate; if there were infective foci (local necrosis), the score was (3) regarded as severe.

Immunohistochemistry technique

Immunohistochemistry was performed to assess TLR4, AKT, phosphoinositide 3-kinases (PI3K), signal transducer and activator of transcription 3 (STAT) in brain tissue. An immunostaining method was used to stain paraffin-embedded slices. Briefly, the sections were deparaffinized in xylazine, followed by rehydration in an arranged ethanol (100-75%), and soaked in distilled water. After that, the sections were exposed to a retrieval buffer in a water bath for about 20 min at 95 ℃, and after removing and cooling for 2 min at room temperature washed by washing buffer 2 multiple 5 min. Then, the slices were incubated in 3% hydrogen peroxide solution to stop endogenous peroxidase action for 5 min and subsequently followed by washing twice with washing buffer.

Subsequently, the sections were incubated with one of the anti-TLR4, AKT, PI3K, and STAT antibodies at concentrations of 1:100 and 1:200 for TLRs, respectively at 4 ℃ overnight, washed 2 multiple 5 min and incubated with conjugated secondary antibody for 1 h, then washed and subjected to horseradish peroxidase for 0.5 h.

The slides were washed 2 times with buffer (5 min for each one), 3,3_diaminobenzidine was then added to the samples and incubated for 8 min and subsequently soaked in distilled water for 2 min. Hematoxylin stain solution was added to the slides for 2 min. Afterward, slides were transmitted to a hot plate (at 40 ℃) till completely dried. The slides were immersed in xylene for 2 min, covered, and put on a hot plate to dry. Finally, the stained slides were observed under the Leica DM750 microscope (Germany) and the protein expression of TLR4, AKT, PI3K, and STAT was computed using the histochemical score technique (range of 0-300) by multiplying the intensity by the percentage of the stained areas. The staining intensity was graded as zero, no staining; 1, mild staining; 2, moderate staining; 3, severe staining. The percentage of stained cells was graded from 0 to 100% (18).

Statistical analysis

The data were analyzed using SPSS version 26. The data are presented as mean ± SD. ANOVA test was performed for multiple comparisons among all groups, followed by Bonferroni mean rank testing. The Kruskal-Wallis test was used to examine the statistical significance of differences across various groups in brain histopathological alterations overall severity score (mean score) and (mean rank) for immunohistochemistry. *P*-values ≤ 0.05 were considered statistically significant for the tests.

RESULTS

Effect of dasatinib on inflammatory markers

The brain levels of IL-6, IL-1β, and TNF-α were considerably higher in sepsis and vehicle groups than in the sham group but these levels were significantly reduced in the dasatinib-treated group (Fig. 1A-C). Brain tissue level of IL-10 significantly increased in sepsis and vehicle groups as compared to the sham group. On the other hand, treatment with dasatinib significantly increased IL-10 levels (Fig. 1D).

Dasatinib downregulated TLR4, AKT, PI3K, and STAT3 expression in brain tissue

The immunohistochemistry expression of TLR4, AKT, PI3K, and STAT3 were much greater in sepsis and vehicle groups in comparison to the sham group. Alternatively, treating with dasatinib considerably decreased the expression of these proteins in the treatment group as compared to sepsis and vehicle groups (Figs. 2-4).

Histopathological examination

Histopathological examination revealed normal brain tissue in the sham group. The cross-section of brain tissue of the sepsis group showed abnormal brain structure and severe brain injury, including increased cellularity (microglia and astrocyte activation), extra invasion of red blood cells, mild inflammation, focal necrosis, and severe edema. In comparison to sepsis and vehicle groups, the dasatinib-treated group revealed limited histological alteration (Fig. 5).

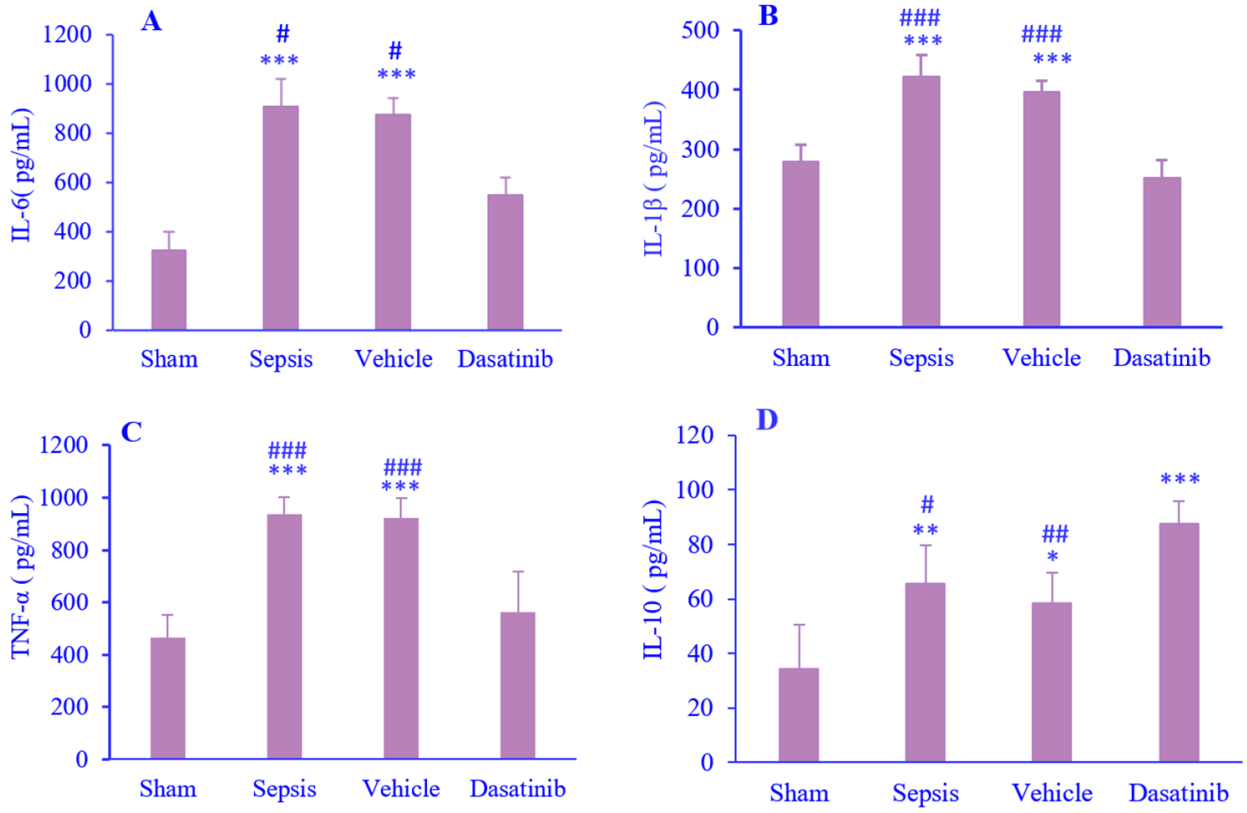

Fig. 1. Effect of dasatinib on inflammatory mediators of brain tissue including (A) IL-6, (B) IL1β, (C) TNF-α, and (D) IL-10. The data are presented as mean ± SD. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ indicate significant differences in comparison with the sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus dasatinib. IL, Interleukin; TNF, tumor necrosis factor-alpha.

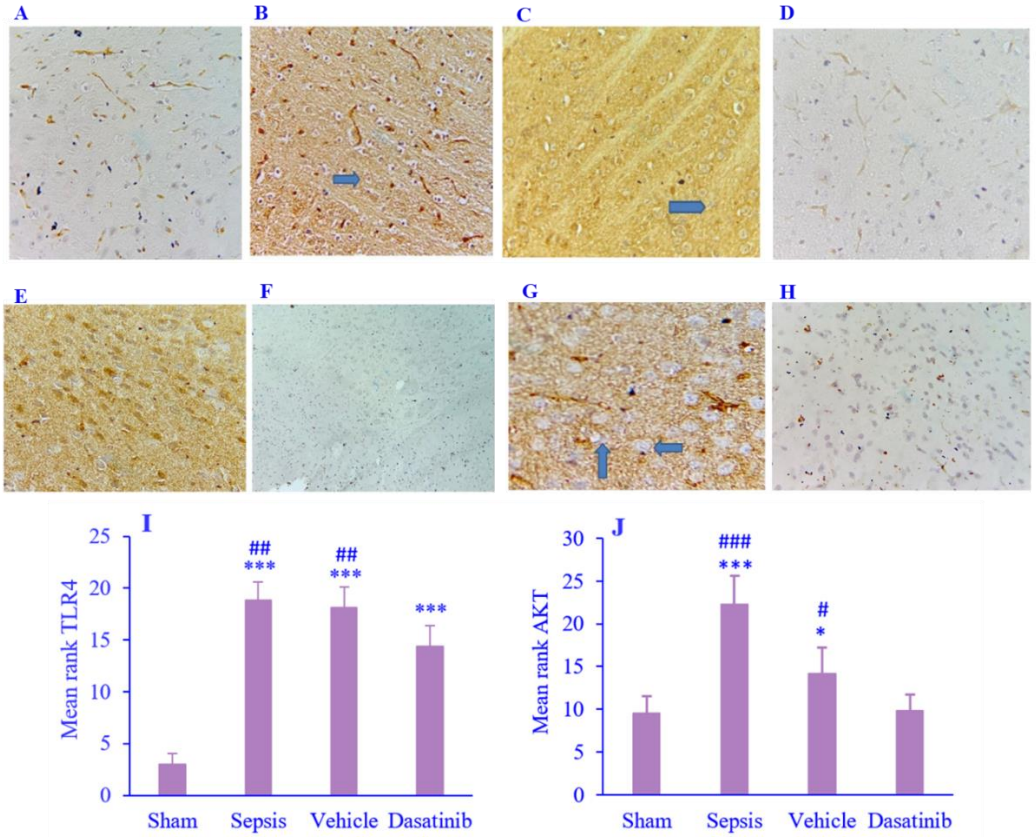

Fig. 2. (A-D) Cross section of the brain showed TLR4 immunohistochemistry; (A) negative sham group; (B) TLR4-positive sepsis group, blue arrow refers to the cytoplasmic stain; (C)TLR4-positive vehicle group, blue arrow refers to nucleus spread staining; and (D) TLR4-negative dasatinib treatment group. (E-H) Cross section of the brain showed AKT immunohistochemistry of the (E) negative-sham group; (F) AKT-positive sepsis group, diffuse cytoplasmic stain and few nuclei stained; (G) AKT-positive vehicle group, blue arrows refer to nucleus spread staining; and (H) AKT-negative dasatinib treatment group. Mean rank of (I) TLR4 and (J) AKT in brain tissue. The data are presented as mean ± SD. $^{*}P < 0.05$ and $^{***}P < 0.001$ indicate significant differences in comparison with the sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus dasatinib. TLR, Toll-like receptor; AKT, protein kinase B.

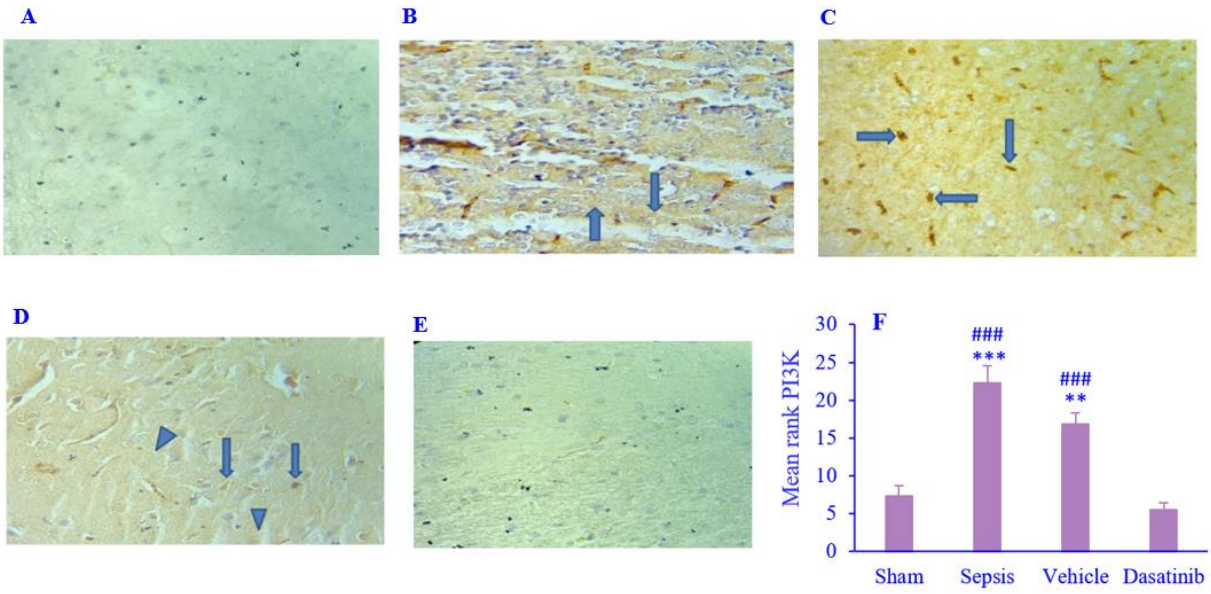

Fig. 3. (A-E) Cross section of the brain showed PI3K immunohistochemistry of the (A) negative sham group; (B) positive-sepsis group, cytoplasmic stain and nuclear spread, blue arrows refer to the cytoplasmic stain; (C and D) positive vehicle group, cytoplasmic and scattered nuclear stain, blue arrows refer to nuclear stain, arrowhead refers to the cytoplasmic stain; (E) negative dasatinib group; (F) mean rank of PI3K in brain tissue. The data are presented as mean ± SD. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate significant differences in comparison with the sham group; $^{\#\#\#}P < 0.001$ versus dasatinib. PI3K, Phosphoinositide 3-kinases.

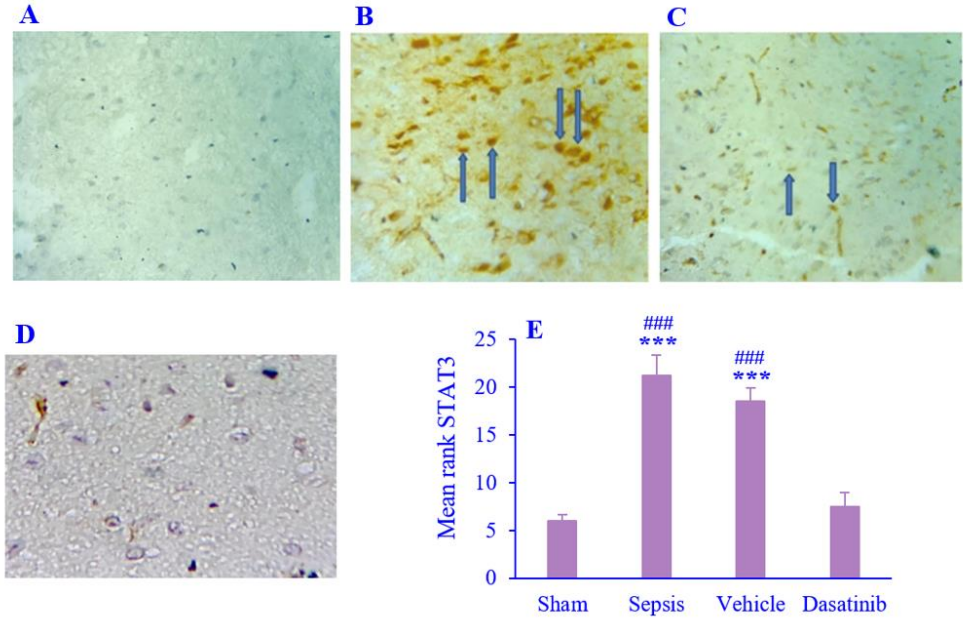

Fig. 4. (A-D) Cross section of the brain showed STAT3 immunohistochemistry of the (A) negative sham group (B) positive sepsis group, blue arrows refer to the nuclear stain; (C) positive vehicle group, blue arrows refer to the nuclear stain; (D) negative dasatinib-treated group; and (E) mean rank of STAT3 in brain tissue. The data are presented as mean ± SD. ***P < 0.001 indicates significant differences in comparison with the sham group; $^{###}P$ < 0.001 versus dasatinib. STAT, signal transducer and activator of transcription 3.

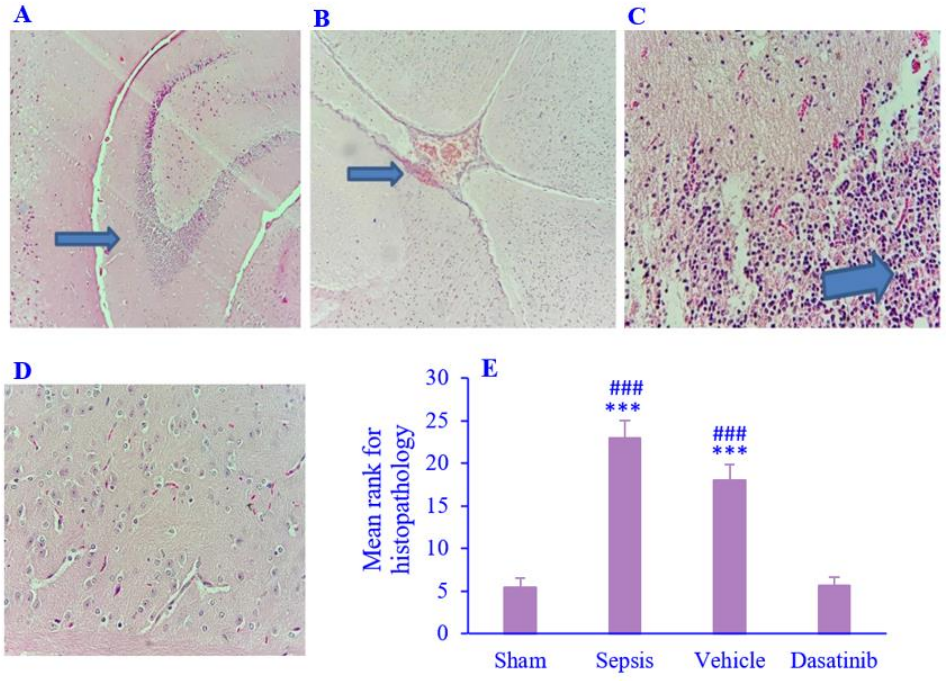

Fig. 5. (A-D) Histopathological examination of the brain. The sections were stained by heamatoxylin and eosin including (A) the sham group scored 0 normal histology, blue arrow refers to the cerebellum; (B) the sepsis group scored 2 changes, edema, increased cellularity, and hemorrhage (blue arrow); (C) vehicle group score 3 changes, area of inflammation (blue arrow) and necrosis; (D) dasatinib-treated group normal histology score 0; (E) mean rank for a histopathological score of brain tissue. The data are presented as mean ± SD. ***P < 0.001 indicates significant differences in comparison with the sham group; $^{###}P$ < 0.001 versus dasatinib.

DISCUSSION

Sepsis is a complicated uncontrolled immune and inflammatory response to infection that leads to multiorgan dysfunction when the host response is amplified and deregulated (19). Sepsis causes acute brain dysfunction that is unrelated to direct brain infection and is shown by a variety of clinical and electroencephalographic abnormalities, known as sepsis-associated encephalopathy (20). It is substantially linked to increased mortality and long-term cognitive deficits (21). Deregulated cytokine responses have an important role in tissue damage and neurological impairments (22,23). Non-resolving neuroinflammation is a potentially hazardous process of brain injury (24). Therefore, preventing the occurrence of sepsis or limiting its harmful impacts has been a long-standing goal of the researchers and the requirement for pharmacological medication to prevent or treat this problem is urgent.

This experimental study reported that there is a significant elevation in TNF-α, IL-1β, IL-6, and IL10 expression in brain tissues in sepsis and vehicle groups in comparison with the sham group after CLP-induced sepsis. Fu *et al.* found that the serum and brain tissue levels of TNFα and IL-1β were elevated 30 min up to 6 h post-LPS administration (25). IL-6 is likely to be an essential mediator for the inflammatory response in septic mice after 24 h from CLP (26). Regarding sepsis, IL-10 possesses anti-inflammatory properties and suppresses pro-inflammatory cytokines generation (27).

According to this study, a significant reduction in TNF-α, IL-1β, and IL-6 in the dasatinib-pretreated group with a substantial rise in anti-inflammatory cytokine (IL-10) levels. This may indicate that dasatinib had a neuroprotective function. Guo *et al.* showed that dasatinib therapy reduced the release of proinflammatory cytokines such as TNFα, IL-1, and IL-6 while increasing the release of IL-10 in patients with arthritis (28).

This research demonstrated that there is a significant rise in brain TLR4 expression in sepsis and vehicle groups as examined by immunohistochemistry. Our findings are in agreement with another research. Mice brain tissue when subjected to CLP, exhibited neuroinflammation. Microglia (the majority of activated immune cells in the central nervous system) excited and triggered an increase in TLR-4 mRNA production. TLR4 upregulation will activate intracellular signaling pathways such as nuclear factor kappa B (29).

Immunohistochemical examination of the dasatinib-treated group demonstrated a significant decrease in TLR4 expression as compared with sepsis and vehicle groups. Dasatinib inhibits the TLR4 signal, modifying the amounts of proinflammatory cytokines generated by LPS and this leads to a reduction in the level of proinflammatory cytokines (11). Another experimental study showed that dasatinib inhibits glial excitation, neutrophil recruitment, and proinflammatory cytokine production in culture after LPS stimulation. This drug also inhibits inflammation by blocking TLR4 and downstream effectors such as ERK and AKT (30).

Our results reported that there is a significant expression of AKT, PI3K, and STAT3 in brain tissues of both the sepsis and vehicle groups. Ryu *et al.* found that the LPS-stimulated AKT or ERK signaling in the brain leads to the production of proinflammatory cytokines. Furthermore, LPS induced nuclear STAT3 phosphorylation (11).

Dasatinib-pretreated mice had a significant reduction in brain tissue levels of PI3, AKT, and STAT3 as compared with the sepsis group. Our findings are consistent with other study research. Dasatinib inhibits the expression of essential oncogenic signaling cascade components such as AKT and STAT3 (11,31).

The present study has shown that sepsis and vehicle groups had a significantly higher degree of brain tissue injury as compared with sham groups. Brain tissues obtained from mice treated with dasatinib had significantly less cellular injury. This result indicated that dasatinib could protect against brain dysfunction. Our data are compatible with those of other experimental studies. Dasatinib improves the intensity of experimental autoimmune encephalomyelitis (mouse model for multiple sclerosis) and reduces

inflammatory cell infiltration into the central nervous system, according to a study conducted on female mice. Dasatinib appears to reduce macrophage/microglia stimulation, TNF-α release, and neuronal ischemia alterations (32).

CONCLUSION

Dasatinib attenuated endotoxemia-induced acute brain damage in mice *via* modulating TLR4, PI3K, AKT, and STAT3 downstream signaling pathways.

Conflict of interest statements

The authors declared no conflict of interest in this study.

Authors' contributions

All authors contributed equally to this work. E. Sabah took responsibility for the integrity of the work as a whole from inception to the published article and should be designated as guarantor.

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