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The Protective Roles of Urinary Trypsin Inhibitor in Brain Injury Following Fat Embolism Syndrome in a Rat Model

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Abstract

Fat embolism syndrome (FES) is a common complication following long bone fracture; fat droplets are released into the blood circulation and form embolisms, mainly in lung and brain. However, the potential mechanisms involved remain to be clarified. In this study, the mechanism of brain injury following FES and the protective effects of urinary trypsin inhibitor (UTI)—a serine protease inhibitor—were investigated. Sixty male Sprague-Dawley rats were divided randomly into sham, FES and FES+UTI treatment groups. The FES model was established using tail vein injection of glycerol trioleate, and UTI was administered by intraperitoneal injection immediately following FES. Brain/lung water content evaluation, Evans blue content and magnetic resonance imaging examination were used to assess the effects of UTI. Furthermore, immunohistochemistry and western blot were also applied to explore the protective mechanism of UTI following FES. The results of oil red O staining indicated that the FES model was successfully established. UTI could significantly attenuate blood-brain-barrier (BBB) disruption, as seen through brain edema evaluation and Evans blue content examination. Immunofluorescence staining results indicated that the TLR4-JNK pathway was involved in brain injury after FES; this effect could be quenched by UTI treatment. Furthermore, UTI could decrease the levels of downstream target proteins of the TLR4-JNK pathway, phosphorylated-NF- κB (p65) and p53 in brain. Our results showed that UTI could alleviate BBB injury after FES through blocking activity of the TLR4-JNK pathway.

Keywords

fat embolism syndrome, urinary trypsin inhibitor, inflammation, apoptosis, rat

Introduction

Fat embolism syndrome (FES) is an embolic disease. Circulating fat can be embolized in several organs, notably in lungs and brain[1,2], and FES is a potentially life-threatening disease[3]. FES can occur following long-bone fractures and fat crushing[2]. The main symptoms of FES include pulmonary injury (tachypnea and dyspnea) and neurological deficits (confusion, drowsiness, or coma), and dermatological lesions (petechial rash)[4].

So far, most studies on FES have focused on lung injury because it is believed that fat droplets in the blood occlude mainly lung capillaries[5]. However, neurological signs, which can precede the development of respiratory symptoms and may be the primary signs[6], are reported in up to 86% of FES patients[7]. Although death induced by FES is commonly attributed to pulmonary insufficiency[8], some patients who die have usually been deeply comatose. The role of brain injury following FES has been considered as a primary cause of death[9]. Although damage to the blood-brain-barrier (BBB, formed by endothelial cells, basement membrane, astrocytes and pericytes) is involved in pathology of FES[10], the specific mechanisms involved still need further investigation.

The mechanism of injury following FES involves a mechanical embolic phase followed by a biochemical response. The mechanical phase includes the blockage of

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capillaries, such as in lungs and brain, which results in venoarterial shunting and hypoxemia. The biochemical phase begins with free fatty acids (FFAs) produced by the fat. FFAs in the lung parenchyma lead to disruption of alveolar capillary membranes, interstitial hemorrhage, and pulmonary edema[11]. However, the damage that FES causes to brain, and its mechanism, has not yet been fully elucidated. Using magnetic resonance imaging (MRI) examination, morphological, and western blot methods, the present study explores capillary injury in brain after FES[12].

Toll-like receptor 4 (TLR4) is a member of the TLR family, which recognize distinct microbial components and mediate innate immune responses for host defense[13,14]. Inappropriate activation of TLR4 and its downstream mitogen-activated protein kinase (MAPK) pathways, e.g., c-jun N-terminal kinase (JNK)[15] will initiate inflammation and apoptosis cascades and lead to severe inflammatory and autoimmune injuries[16].

Urinary trypsin inhibitor (UTI) is a Kunitz-type protease inhibitors found in human urine and synthesized from inter-α-trypsin inhibitor[17]. UTI has been used for treatment of acute pancreatitis and circulatory shock[18]. UTI can inhibit plasmin, chymotrypsin, and neutrophil elastase as well as trypsin[19,20]. It can suppress the production of TNF-α from lipopolysaccharide (LPS)-activated macrophages[21]. It is also reported that UTI can protect the BBB integrity through blocking the inflammatory and apoptotic pathway after subarachnoid hemorrhage[22]. Whether UTI can also protect brain tissue from FES injury, and the mechanism of such protection, still need further study.

In this study, the roles of UTI in maintaining BBB integrity after FES were explored, and the potential mechanisms were also investigated. The results indicated that UTI could protect the BBB through blocking inflammatory and apoptotic cascades in capillaries following FES.

Materials and Methods

All procedures were performed following protocols approved by the Association of Medical Ethics of Peking University Health Science Center in Beijing, China.

Establishment of FES Model

The whole study design is shown in Fig 1. Male Sprague-Dawley rats (280–300 g) were purchased from Peking University Health Science Center. The animals were allowed free access to food and water, and housed in 12-h dark/light cycle in a temperature- and humidity-controlled environment.

The animals were divided randomly into three groups: (1) Sham group ($n = 20$); (2) FES+NS (normal saline) group ($n = 20$); (3) FES + UTI group ($n = 20$).

The rats were anaesthetized by intraperitoneal administration of pentobarbital sodium (50 mg/kg; Abbott Laboratory, North Chicago, IL, USA). The FES model was established by intravenous injection of 0.2 ml triolein (1,2,3-tri[cis-9-octadecenoyl]glycerol, CAS: 122-32-7, Sigma, St. Louis, MO, USA) into the caudal vein. In sham group, the rats were intravenously administered 0.2 ml NS. In the FES + UTI group, UTI (50,000 U/kg, Techpool Biopharma Co., Ltd, China) was administered intravenously 30 min after administration of triolein, as reported previously[22,23].

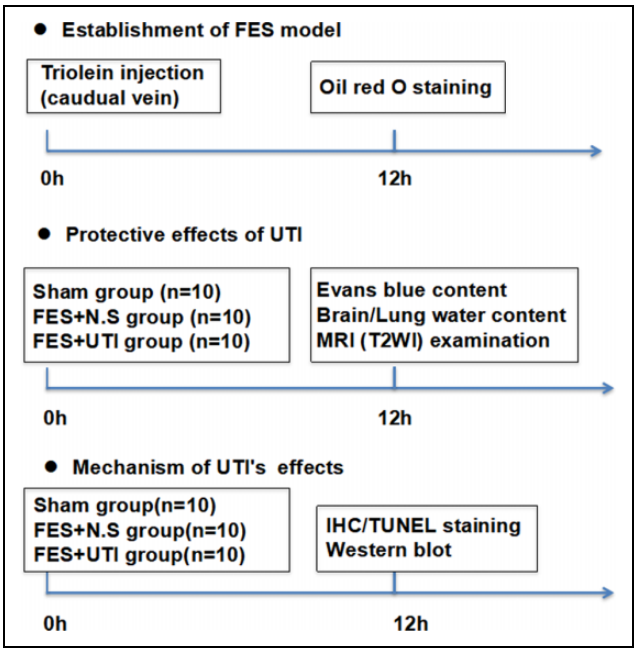

Fig 1. Experimental designs. (A) Establishing the rat FES model and confirming its validity; (B) Detecting the protective roles of UTI using Evans blue content, water content, and MRI examination; (C) Exploring the potential mechanism of protective effects of UTI. UTI: urinary trypsin inhibitor; FES: fat embolism syndrome; IHC: immunohistochemical staining; TUNEL: terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling staining.

Water Content Measurement

The animal's brain and lungs were harvested at 12 h following FES. The brain was divided into left hemisphere, right hemisphere, cerebellum and brainstem, as previously described[24]. The specimens were weighed immediately (wet weight) and weighed again after drying in an oven at 105°C for 72 h (dry weight). The water content was calculated using the following formula: ([wet weight – dry weight]/wet weight) × 100%.

Magnetic Resonance Imaging

MRI (T2 weighted image, T2WI) was performed as reported by others[25]. In this study, the hippocampus is the region of interest (ROI) due to its higher sensitivity to hypoxia or edema than other brain regions following FES.

At 12 h following FES, the anesthetized rat was placed on a special frame in supine position, and introduced into a 3.0-Tesla MRI animal scanner (Siemens, Munich, Germany). The head of the rat was set in a "birdcage coil" (diameter = 30 mm). A T1-weighted image sequence was conducted for orientation of subsequent scans. A muscle relaxant was applied continuously via the femoral artery to minimize movement artifacts (diluted atracurium besilate, initial bolus 4 mg/kg followed by 4.5 mg/h; Glaxo Wellcome, Brentford, UK).

T2WI was performed using the following parameters: repetition time (TR) = 2500 ms; echo time (TE) = 8.1–65.6 ms; flip angle = 90°; matrix = 0.63 mm × 0.63 mm; slice thickness = 2 mm; number of averages = 1; echo train length 1. The data sets of T2WI consisted of eight consecutive, 2-mm thick slices without slice gap. All process was conducted by two experienced, independent radiologists. The T2 values of hippocampus each group were recorded and analyzed.

Immunohistochemical Staining

Immunohistochemical staining was performed as reported previously by others[26]. The brains or lungs were fixed by cardiovascular perfusion with 0.01 mol/L PBS and 4% paraformaldehyde (PFA), and then postfixed in 4% PFA followed by 30% sucrose. The coronal sections of 10 μm thickness containing the bilateral hippocampus were cut using a cryostat (Leica Microsystems, Bannockburn, IL, USA) and mounted onto poly-L-lysine-coated slides. Four series of sections were incubated with following primary antibodies: mouse anti-phosphorylated NF-κB (p65), rabbit antiphosphorylated p53 (Cell Signaling Technology, Danvers, MA, USA), and mouse anti-TNF-α, rabbit anti-cleaved caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The sections were then treated with the corresponding species ABC Kit (Santa Cruz Biotechnology). Peroxidase activity was detected using 3-diaminobenzidine (DAB) and H_2O_2 for 5 min. The sections were dehydrated and coverslipped.

The sections for immunofluorescence staining were incubated overnight with primary antibodies for TLR4 and phosphorylated JNK (Cell Signaling Technology). The sections were treated with the corresponding fluorescence labeled second antibodies(1:200, Santa Cruz Biotechnology). The results were observed with the microscope (Olympus BX51, Tokyo, Japan). Serum was applied instead of primary antibody as the negative control.

Oil Red O Staining

Frozen tissue sections (10μm thickness) were stained for 5 min with a filtered solution of 1% oil red O in 60% aqueous triethylphosphate and then mounted in glycerin jelly, as reported previously[27].

TUNEL Staining

The apoptosis of endothelial cells was observed using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) method. Brain tissue sections were stained using a TUNEL Kit (Roche, New York, NY, USA), and TUNEL-positive cells were revealed by fluorescein-dUTP with dNTP and POD with DAB as described previously[28].

Western Blot

Western blotting was performed as reported by others[29]. Rats under deep anesthesia were perfused transcardially with 250 mL 0.1 mol/L PBS (pH 7.4). The bilateral hippocampi were harvested and homogenized in RIPA lysis buffer (Santa Cruz Biotechnology) followed by centrifugation at 14,000 *g* at 4°C for 30 min. The protein concentration of the supernatant was measured using a detergent compatible assay (Bio-Rad, Hercules, CA, USA). The protein sample (40 μg) was loaded on a Tris glycine gel, electrophoresed, and transferred on a nitrocellulose membrane, and then blocked with blocking solution and incubated with primary antibody overnight at 4°C. The primary antibodies (1:1000) were mouse antiphosphorylated NF-κB (p65), rabbit antiphosphorylated p53 (Cell Signaling Technology), and mouse anti-TNF-α, rabbit anti-cleaved caspase-3 (Santa Cruz Biotechnology) . Nitrocellulose membranes were then incubated with the corresponding secondary antibodies (1:2000, Santa Cruz Biotechnology) for 1 h. The bands were probed with a chemiluminescence reagent kit (Amersham Bioscience, Arlington Heights, IL, USA) and quantified by densitometry with Image J software (National Institutes of Health, Bethesda, MD, USA). β-Actin was also blotted on the same membrane as the loading control.

Statistical Analysis

All data were expressed as mean ± standard deviation (SD), and were analyzed by one-way analysis of variance (ANOVA) with the Tukey-Kramer post hoc tests using Sigmastat software (version 4.0, Systat, San Jose, CA, USA). A P-value of less than 0.05 was considered statistically significant.

Results

Establishment of FES Model

First, we evaluated the validity of the FES model in this study. The results of oil red O staining showed that there are numerous fat droplets distributed in the capillaries of both lungs and brain (Fig 2A1, B1, A2, B2). These indicated that fat droplets from caudal veins embolized successfully in the brain capillaries, and played their mechanical and biochemical roles. Additionally, we used Evans blue as an albumin

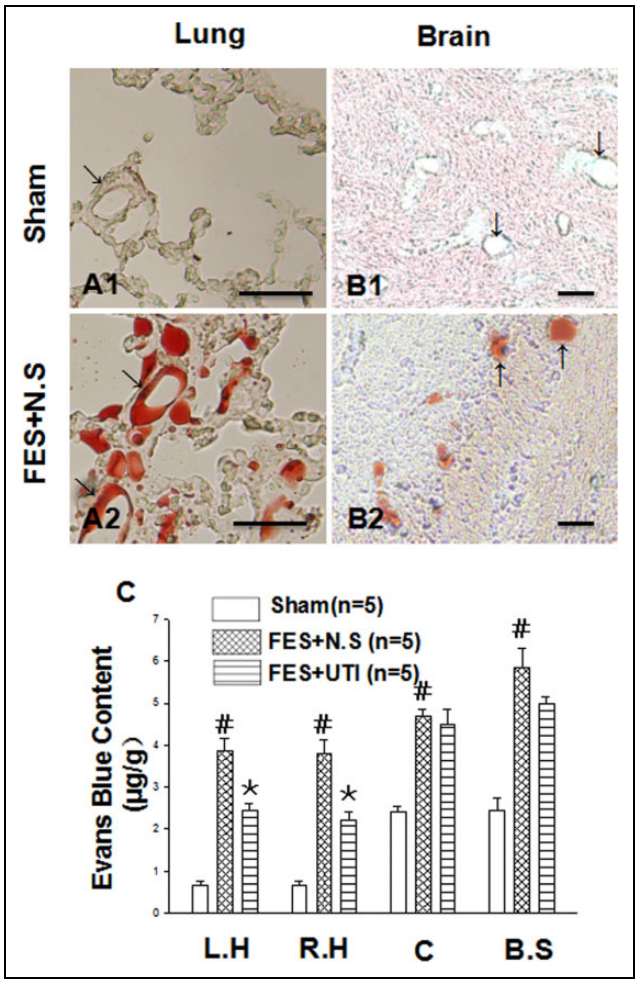

Fig 2. Oil red O staining and Evans blue content evaluation. At 12 h following FES, there are numerous fat emboli (red staining) distributed in the capillaries of lungs (A1 and A2) and hippocampus (B1 and B2). Additionally, the Evans blue content in whole brain was significantly increased following FES, and UTI treatment could markedly reduce the amount of Evans blue in the left hemisphere and right hemisphere (C). Arrows in (A1, B1, A2, B2) indicate the lumens of capillaries; in (A1, A2), scale bar = 100 μm, in (B1, B2), scale bar = 20 μm. #in (C), $P < 0.05$ compared with that of Sham group; *$P < 0.05$ compared with that of FES+NS group. L.H: left hemisphere; R.H: right hemisphere; C: cerebellum; B.S: brain stem.

indicator in blood to assess BBB permeability. Normally, Evans blue cannot infiltrate into the brain parenchyma owing to BBB resistance. However, in the FES model, the Evans blue content in the hippocampus increased markedly, which revealed BBB injury in the brain following FES (Fig 2C). Furthermore, the water content in lungs and brain was also significantly increased as shown by water content evaluation and MRI (T2WI) (Fig 3A, B, C). All the above results confirmed the validity of the rat FES model used in this study. This is an indispensable prerequisite for exploring the injury mechanism and effects of UTI following FES in this study.

The Protective Effects of UTI

After UTI treatment, the Evans blue content in the hippocampus decreased markedly (Fig 2C). In addition, the water content in brain and lungs also declined significantly following UTI treatment (Fig 3A, B). Furthermore, MRI (T2WI) results also showed that the UTI could alleviate edema in bilateral hippocampi (Fig 3C). These results indicated that UTI played an important protective role in maintaining BBB integrity following FES; however, the specific mechanism still needed further investigation.

Mechanism of UTI Effects

Since fat droplets were distributed in the lumens of brain capillaries, we observed pathological lesions of endothelial cells following FES. The immunofluorescence staining results revealed that TLR4 and p-JNK were both activated in endothelial cells following FES, and UTI treatment could significantly quench their activities (Fig 4). The immunohistochemistry staining results showed that downstream pathway proteins, such as NF-κB (p65), TNF-α, p53, and cleaved caspase-3, in endothelial cells were increased after FES, an effect that could be attenuated by UTI treatment (Fig 5). Additionally, TUNEL staining results showed that UTI treatment could alleviate endothelial cell loss through blocking the apoptosis cascade following FES (Fig 5). Furthermore, western blot results showed that expression levels of NF-κB (p65), TNF-α, p-p53, and cleaved caspase-3 in hippocampi were also enhanced, which could be downregulated by UTI administration (Fig 6).

Discussion

In this study, we explored the potential mechanism of brain injury following FES, and the effects of UTI in alleviating injury. The results indicated that, after FES, there was BBB disruption due to the loss of endothelial cells induced by inflammation and apoptosis cascades. UTI, as an α-trypsin inhibitor, played protective roles in maintaining BBB integrity through its anti-inflammation and anti-apoptosis effects.

FES is a clinically significant problem, particularly after bone fractures and joint replacements. Respiratory dysfunction, neurological symptoms, and a petechial rash are the major diagnostic features for FES. Neurological signs have been reported in up to 86% of patients[7], and may be the primary cause of death[30].

There are two opinions as to the cause of FES. One theory maintains that the intramedullary fat is absorbed into the circulation as emboli after a fracture[31]. The other believes that FFAs are released when lipase produced by the lungs acts on embolic fats. FFAs are toxic to pulmonary or brain endothelial cells[31], and can lead to interstitial and alveolar hemorrhage, edema, and even acute respiratory distress syndrome (ARDS)[32].

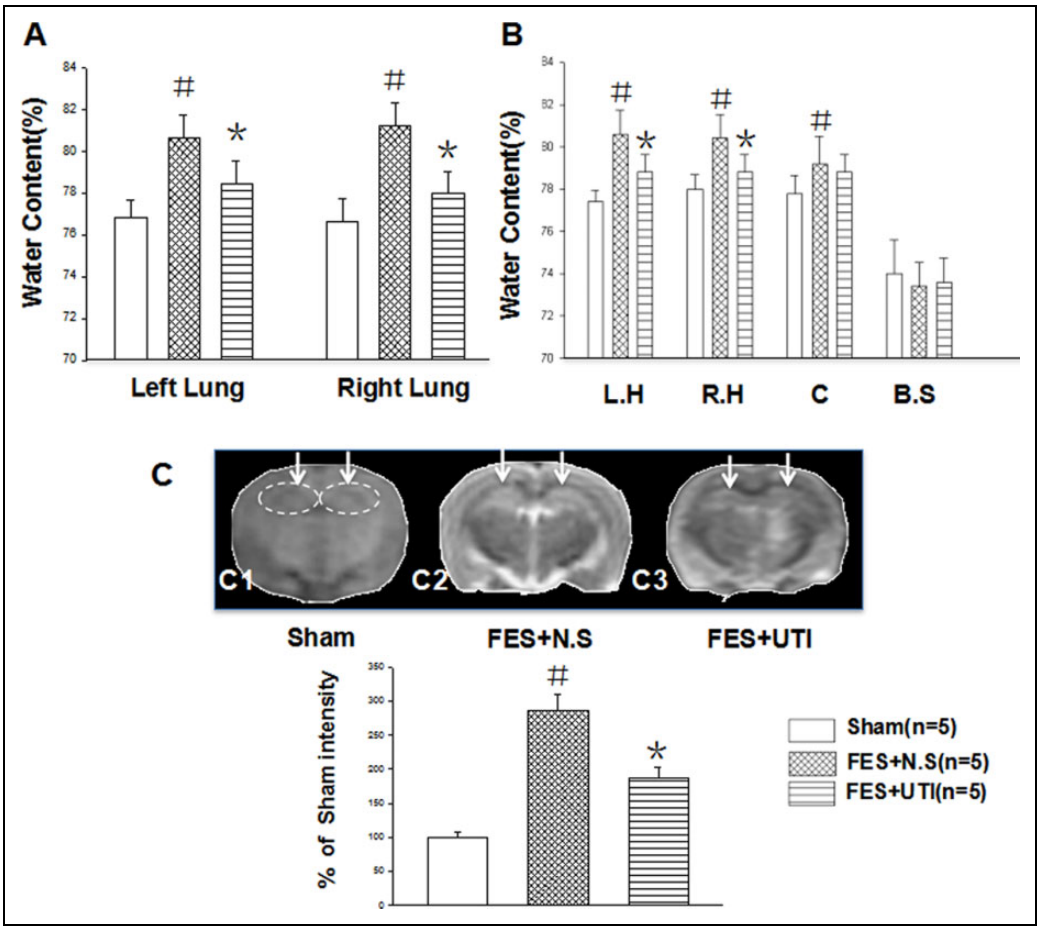

Fig 3. The water content evaluation in lungs and brain. At 12 h following FES, the water contents in lungs and brains were significantly increased; after UTI treatment, the water contents in lungs, left hemisphere and right hemisphere were markedly reduced (A, B). Furthermore, the MRI T2WI results also indicated that there was vasogenic edema in the bilateral hippocampi of brain, which could be alleviated by UTI administration(C). # $P < 0.05$ compared with that of Sham group; *$P < 0.05$ compared with that of FES+N.S group. L.H: left hemisphere; R.H: right hemisphere; C: cerebellum; B.S: brain stem.

There are two types of injury following FES: a mechanical embolic phase, and a biochemical or metabolic response. The mechanical phase involves blockage of capillaries, resulting in hypoxemia, and hypoperfusion in the lungs or brain. The biochemical damage begins after the capillaries filter fat droplets[11]. Lipoprotein lipase, secreted by endothelial cells in the capillary walls, the fat and triglycerides into FFAs, which are toxic to the endothelial cells of organs.

There are three methods to establish the FES model, one is tail vein injection of triolein; the second is to take body fat and inject it into the femoral vein; finally, there is a method of artificial fractures to establish the model[5,33,34]. In this study, we used the method of triolein injection from the caudal vein, using oil O staining (a specific method to detect fat)[35]. The fat emboli were found distributed in lungs and brain, which indicated that the rat FES model used in this study could accurately simulate the clinical FES situation; therefore, reliable data about the injury mechanisms of FES and the effects of UTI could be obtained in this study.

So far, greater attention has been paid to lung damage after FES; however, brain injury is a common symptom following FES. Although it is reported that BBB injury occurs after FES[10], the mechanism of pathogenesis is not fully understood. In this study, we measured the water content and Evans blue content in the brain. The brain water content was significantly increased after FES; furthermore, the Evans blue content in the hippocampus also increased markedly. These results showed that there is definite vasogenic edema in brain tissue induced by BBB disruption after FES.

Additionally, MRI T2WI was also applied to explore edema after FES, since this method can sensitively detect the vasogenic edema resulting from BBB disruption[36]. The T2 signal intensity increased markedly following FES, which indicated that vasogenic edema induced by BBB injury occurred in brain tissue.

Since there was some evidence of BBB disruption after FES, we next explored the pathological mechanism of BBB

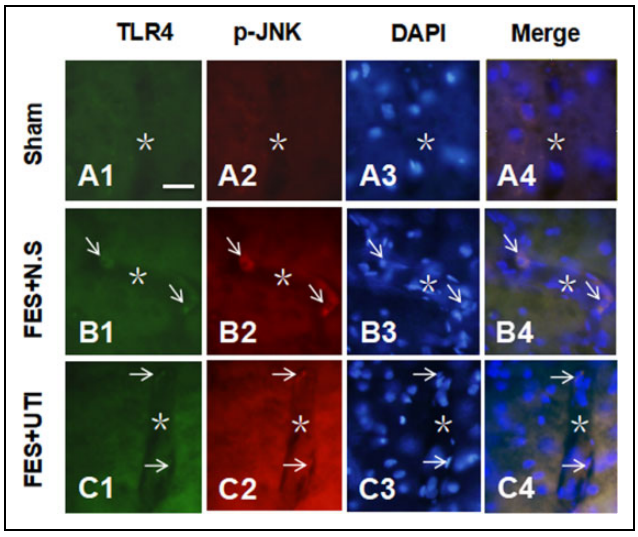

Fig 4. Immunofluorescence staining in brain following FES. The results indicated that both TLR4 and p-JNK were activated in the endothelial cells following FES(A1–B4), which could be significantly quenched by UTI treatment (C1-C4). *indicates the lumen of capillaries; arrows indicate endothelial cells, scale bar = 10 μm.

damage. The BBB is composed of blood vessels whose endothelial cells display specialized tight junctions and extremely low rates of transcellular vesicular transport (transcytosis)[37]. The BBB can block some macromolecules from entering the brain, and it controls substance influx and efflux[37]. In this study, after FES, TUNEL staining showed that apoptotic endothelial cells were increased markedly, which could result in BBB disruption and dysfunction, e.g. edema.

To clarify the mechanism of endothelial cell apoptosis, we performed immunohistochemical staining of hippocampus capillaries. The immunofluorescence staining results revealed that the TLR4-JNK pathway was activated following FES. The evolutionarily conserved TLR family can recognize distinct microbial components and mediate innate immune responses for host defense[13,14]. TLR4 are expressed constitutively in bone-marrow-derived cells and in a number of mucosal cells. A number of studies have demonstrated that TLR4 plays a key role in the mucosal inflammation caused by ischemia/reperfusion (I/R) in hepatic I/R[38], intestinal I/R[39], cardiac I/R[40], and hemorrhagic shock[40]. Activation of TLR4 can stimulate MAPK pathways, e.g., JNK[15], which lead to activation of NF-κB and p53 pathway, and initiate the inflammation and apoptosis pathologies[41–43].

The immunohistochemical staining indicated that NF-κB (p-p65), p-p53, TNF-α, and cleaved caspase-3 in endothelial cells were enhanced, which indicated that the inflammation and apoptosis cascades were activated within the capillaries following FES. Additionally, the western blot results also confirmed these findings. Therefore, there was obvious BBB disruption in the brain after FES, which might be an important mechanism responsible for brain injury.

UTI is a protease inhibitor purified from human urine. UTI can inhibit the activities of proteases such as trypsin,

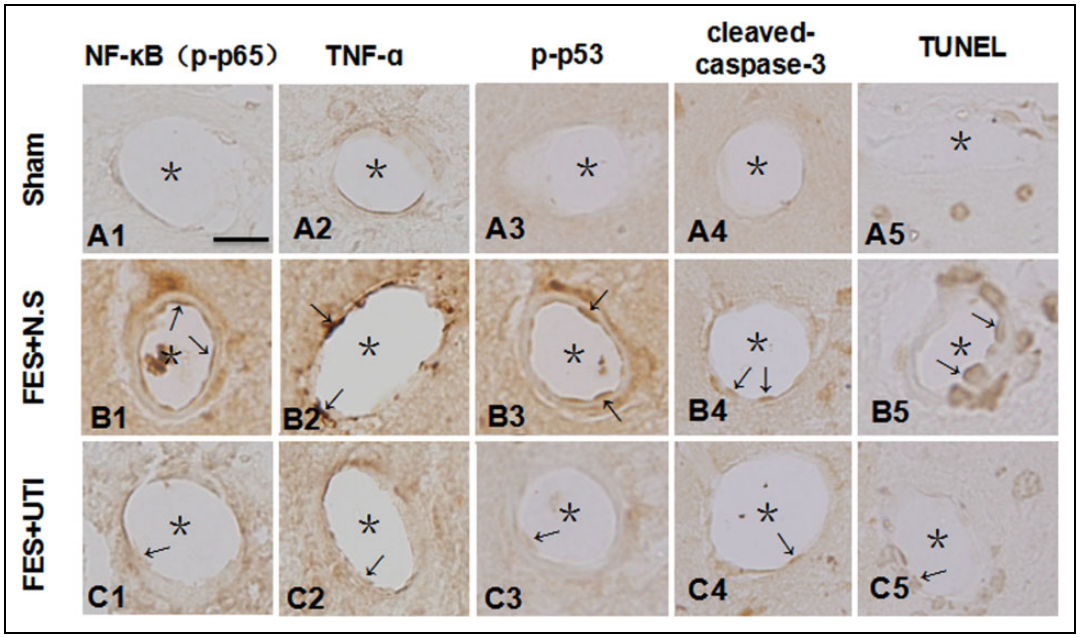

Fig 5. Immunohistochemical staining of endothelial cells following FES. The expression levels of NFκB(p65), TNF-α, p53 and cleaved caspase-3 of endothelial cells in the capillaries were markedly increased (A1–A4, B1–B4), which could be significantly reduced following UTI treatment (C1–C4).In addition, TUNEL staining results indicated that the apoptosis of endothelial cells could be attenuated after UTI administration (A5–C5). *indicates the lumen of capillaries; arrows indicate endothelial cells, scale bar = 20 μm.

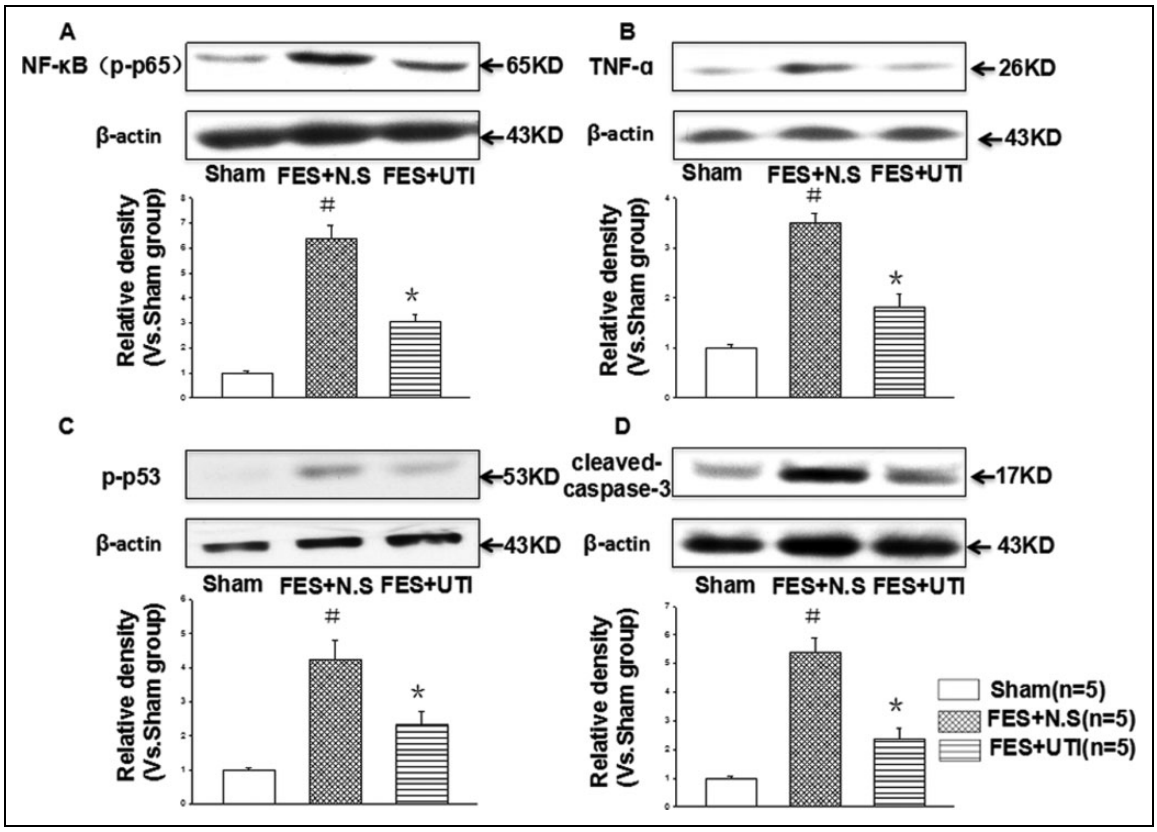

Fig 6. Expression levels of inflammation and apoptotic cascades after FES. The protein expression levels of NF-κB (p-p65)(A), TNF-α (B), p-p53 (C), and cleaved caspase-3 (D) in hippocampus were significantly enhanced after FES, which could be markedly decreased after UTI treatment (A–D). # $P < 0.05$ compared with that of Sham group; * $P < 0.05$ compared with that of FES+N.S group.

chymotrypsin, and elastase. It can also stabilize lysosomal membrane, and suppress the release of lysosomal enzymes[21,44]. Additionally, UTI can reduce the production of cytokine-induced neutrophil chemoattractants and decrease neutrophil accumulation, and thereby alleviate neutrophil-induced endothelial injury[45]. UTI can block the activation of inflammation and apoptosis pathways[46,47]. It is reported that UTI can attenuate reperfusion injury in the ischemic intestine[48], liver[49], and kidney[50]. Furthermore, it can decrease TNF-α production in the rat I/R liver[51]. It is also reported that UTI can inhibit the apoptosis of endothelial cells[22,46]. However, the effects of UTI on maintaining BBB integrity following FES has not yet been studied. In this study, UTI significantly reduced brain water content and Evans blue content after FES, and T2WI results also showed UTI can alleviate brain edema. These results indicated that UTI treatment could attenuate BBB injury following FES.

After observing the protective roles of UTI on BBB, we next explored the potential mechanism of UTI's effects. The results of immunofluorescence staining showed that UTI could quench the activity of the TLR4-JNK pathway in endothelial cells. Additionally, after UTI treatment, the expression level of downstream targets in the TLR4-JNK pathway, such as inflammatory factor NF-κB(p-p65), TNF-α, apoptotic factor p-p53, and cleaved caspase-3, were significantly decreased. More importantly, TUNEL-positive-staining endothelial cells were decreased, too. These results implied that the protective effects of UTI on BBB integrity might be through attenuating inflammation and apoptosis via blocking activities of the TLR4-JNK pathway and its downstream targets.

Conclusion

In this study, we found that BBB disruption might be one of primary brain injuries following FES due to fat emboli in capillaries. UTI could play its protective roles in maintaining BBB integrity through alleviating apoptosis and inflammation cascades in endothelial cells via suppression of the TLR4-JNK pathway.

Ethical Approval

This study was approved by the Association of Medical Ethics of Peking University Health Science Center in Beijing, China.

Statement of Human and Animal Rights

This article contains animal studies approved by the Association of Medical Ethics of Peking University Health Science Center in Beijing, China.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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