Original Article
Treatment with etanercept attenuates brain death-associated liver injuries in rats

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Abstract: Background: Brain death (BD) induces a systemic inflammatory response, resulting in poor graft quality in liver transplantations. TNF-α mediates inflammation after BD and initiates the inflammatory cascade during BD-induced liver damage. In the present study, the therapeutic potential of etanercept, an FDA-approved TNF-α inhibitor, was investigated in a model of BD-induced liver injury. Methods: Using a rat model of BD-induced liver injury, potential protective effects of etanercept were evaluated. The rats were randomly assigned to four groups, including sham + saline, sham + etanercept, BD + saline, and BD + etanercept groups. Liver injury, serum and hepatic TNF-α levels, mRNA expression of inflammatory mediators, liver inflammatory infiltration, and apoptosis were evaluated at 6 hours after BD or sham-operation. Results: Etanercept administration neutralized increased TNF-α levels in the liver and plasma induced by BD. It resulted in noticeable downregulation of *TNF-α, IL-1β, VCAM-1,* and *ICAM-1* mRNA expression and a trend of alleviated inflammatory infiltration. Etanercept administration also resulted in a significant improvement in apoptosis, evidenced by a dramatic decline in levels of cleaved caspase-3 and TUNEL-positive cells, compared to those in BD + saline rats. These changes were accompanied by an amelioration of liver injuries. Conclusion: Current results suggest that etanercept provides protective effects against BD-induced liver injuries in rats. Accordingly, inhibition of TNF-α by etanercept is a promising strategy, protecting the liver quality of brain-dead donors before organ procurement.

Keywords: Brain death, liver injury, inflammatory response, TNF-α, etanercept

Introduction

Donation after brain death (BD) is a major source of liver transplantation. However, BD, recognized as a catastrophic systemic event, induces various pathophysiological processes. These have a negative impact on the liver quality of donors prior to procurement [1]. Animal and clinical studies have also shown that livers originating from donors of BD have worse outcomes after transplantation and decreased long-term graft survival, compared to livers from living donors [2, 3].

Weiss et al. [2] demonstrated that the systemic inflammatory response elicited by BD and characterized by increased circulating inflammatory cytokines is partially responsible for deteriorating donor liver quality and poor outcomes after transplantation. This systemic inflammatory response, triggered by BD, occurs through sev-

eral mechanisms, including hormonal and metabolic derangement, secondary to the catecholamine storm. This leads to ischemia and cytokine release, as well as inflammatory mediators released from the ischemic cerebrum [4]. In the systemic inflammatory response during BD, hepatocytes are exposed to a number of cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 [5, 6]. These cytokines drive inflammatory cells into the liver and activate hepatic Kupffer cells, leading to a local inflammatory response culminating in cellular apoptosis.

TNF-α is the main inflammatory mediator after BD [7]. TNF-α is a potent cytokine with pleiotropic inflammatory and immunological functions via activation of downstream signals by interacting with two cognate membrane receptors (TNFRI and TNFRII). It plays a major role in hepatic inflammation by triggering a cascade of

other cytokines that cooperate in recruiting inflammatory cells, killing hepatocytes, and initiating the wound-healing response [8]. Venkateswaran et al. found that high TNF-α levels were associated with donor cardiac dysfunction [9]. Azarpira and his colleagues also found that elevated plasma TNF-α and IL-6 concentrations before liver procurement are associated with more complications in liver transplantation recipients [10]. During BD, TNF-α not only directly participates in the inflammatory process, but is also involved in the apoptotic process [5].

Etanercept, an FDA-approved TNF-α antagonist, is a recombinant protein composed of a dimer of extracellular domains of human TNF-R2 fused to the Fc portion of human IgG1. It reduces the biological effectiveness of TNF-α by directly binding to TNF-α. It has been used to ameliorate rheumatoid arthritis and ankylosing spondylitis [11-13]. It was also introduced as a part of the immunosuppression protocol for patients with hematopoietic stem cell transplantation or islet cell transplantation [14, 15]. Recent studies have suggested that administration of etanercept may attenuate brain injuries [16, 17], as well as acute liver injuries [18, 19]. Thus, the current study hypothesized that TNF-α blockage using etanercept may attenuate BD-associated liver injuries. The present study, therefore, investigated whether the quality of the liver could be improved by etanercept treatment immediately after BD.

Materials and methods

Animals

Adult male Sprague-Dawley rats, weighing 200 to 300 g, were purchased from the Center of Experimental Animals at the Chinese Academy of Military Medical Sciences (Beijing, China). The rats were housed in a standard environment, with free access to food and water. This experiment was approved by the Animal Ethics Committee of Chinese PLA General Hospital.

BD induction

After anesthesia by isoflurane inhalation, each rat was placed in a supine position for neck surgery. A blunt-tipped PE-50 cannula, attached to a TSD-104A transducer, was inserted into the left carotid artery to continuously record mean blood pressure (MAP) and heart rates (HR) (Biopac Systems Inc., Santa Barbara, CA, USA).

Endotracheal intubation was performed via tracheotomy using a 20 G cannula. This was followed by connection to a small animal ventilator (ALV-8S type ventilator; Alcott Biotech Co., Shanghai, China) with a respiration rate of 80 bpm and tidal volume of 2 mL.

The rats were placed in a prone position after suturing the neck incision. A properly-sized burr hole was drilled at the left frontoparietal area after a scalp midline incision was made. A 4F Fogarty balloon catheter (Edwards Lifesciences Co., Irvine, California, USA), connected to a syringe pump (KD Scientific co.; Holliston, MA, USA), was inserted into the intracranial space via this burr hole. BD was induced by injecting saline into the balloon catheter at a speed of 40 µl/min and under the control of the syringe pump. Once spontaneous respiration ceased, saline injection was terminated. BD was confirmed by physical signs of apnea, maximally dilated and fixed pupils, and the absence of corneal and pedal reflexes. Anesthesia was discontinued after diagnosis of BD. The follow-up period began at the point of apnea detection in rats with BD and at 20 minutes after the point of catheter intubation in sham control animals. The balloon was kept inflated during the entire follow-up period. Sham surgery rats underwent the same surgical procedures, without balloon inflation. Normal saline (0.9%) was used for volume resuscitation. Body temperatures were maintained at 37°C using a heating pad coupled to a rectal probe. Animals with MAP <60 mmHg for a duration of at least 10 minutes after BD were excluded from this study. Rats were sacrificed at 6 hours, obtaining blood and liver samples for further analyses.

Experimental groups

The rats were randomly divided into the following four groups (n=8 per group): (1) Sham + saline group: Rats were subjected to sham surgery and administered 0.9% normal saline (2 mL/kg; iv.) immediately after surgery; (2) Sham + etanercept group: Rats underwent sham surgery and were administered etanercept (5 mg/kg; iv.) immediately after surgery; (3) BD + saline group: Rats underwent BD induction and were administered 0.9% normal saline (2 mL/kg; iv.) immediately after BD was established; (4) BD + etanercept group: Rats underwent BD induction and were administered etanercept (5 mg/kg; iv.) immediately after BD was established.

Etanercept (25 mg) was dissolved in 10 mL saline (2.5 mg/mL), then was stored at 4°C for no more than two days. Etanercept or saline was administered by intravenous right femoral vein injections.

Serum transaminase and TNF-α analysis

Blood plasma samples were centrifuged at 4°C and placed at -80°C for preservation. Serum levels of alanine transaminase (ALT) and aspartate transaminase (AST) were determined at the Clinical Chemistry Laboratory of the Chinese PLA General Hospital, according to standard procedures.

TNF-α concentrations in plasma were measured using a rat TNF-α Enzyme-linked Immunosorbent Assay (ELISA) Kit (Uscn Life Science Inc., Wuhan, China), according to manufacturer protocol.

Histopathology and immunohistochemistry

Liver tissues were fixed by immersion in 10% buffered formalin for three to five days, then embedded in paraffin. Sections of 5 μm were cut and stained with hematoxylin and eosin (H&E).

Immunohistochemistry was performed on 5 μm sections of paraffin-embedded liver tissues, analyzing myeloperoxidase (MPO) (Thermo Scientific, Waltham, MA, USA) expression by development with 3, 3'-diaminobenzidine (DAB), producing a brown color followed by counter staining with hematoxylin. MPO immunostaining was assessed to reflect hepatic neutrophil infiltration, which was used as a marker of inflammation. For each section, MPO positive cells per field were counted by two researchers in 10 adjacent nonoverlapping visual fields at a magnification of ×400. Results are presented as the number of positive cells per field. Negative controls were incubated with phosphate-buffered saline (PBS). Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA) was used for semi-quantitative analysis.

Immunofluorescence analysis of TNF-α and cleaved caspase-3 expression in the liver

Analyzing TNF-α expression and cleaved caspase-3 expression in the liver, an *in situ* immunofluorescence (IF) staining assay was used. Cryosections (8 μm) of the liver tissues were fixed with acetone and washed in phosphate-

buffered saline (PBS) for 5 minutes. This was repeated twice, then they were incubated with 1% bovine serum albumin in PBS for 30 minutes at 20-30°C. The sections were incubated overnight at 4°C using a TNF-α polyclonal antibody (dilution, 1:200; Sigma-Aldrich, USA) and cleaved caspase-3 monoclonal antibody (dilution, 1:200; Cell Signaling Technology, Inc., USA). After the samples were washed with PBS three times (5 minutes each), fluorescence secondary antibodies (diluted at 1:400) were dropped onto the sections in the dark. The sections were incubated for 1 hour in a wet box at 37°C. Afterward, the sections were washed with PBS (pH 7.4) for 5 minutes. This was repeated twice. They were then stained with 4',6'-diamidino-2-phenylindole (DAPI) for 10 minutes in dark conditions. Finally, the sections were sealed with buffered glycerol and images were obtained under a fluorescence Imaging microscope System (Olympus Corp.; Tokyo, Japan). Cleaved caspase 3-positive cells were counted in 10 random 400× viewing fields using Image-Pro Plus 6.0. Results are expressed as the percentage of cleaved caspase 3-positive cells per total hepatic cells in each section.

In situ apoptosis assay

Sections (5 mm thick) were cut from paraffin-embedded rat livers. The TUNEL technique was used to determine apoptosis *in situ* using a TACS® TdT-DAB *in situ* Cell Death Detection Kit (TREVIGEN, Gaithersburg, MD, USA), according to manufacturer instructions. Positively stained apoptotic cells in the liver were counted randomly in 10 random 400× viewing fields using Image-Pro Plus 6.0. Results are expressed as the percentage of TUNEL-labeled cells per total hepatic cells in each section.

Semi-quantitative RT-PCR

Total RNA was isolated from liver samples using the RNA simple Total RNA Kit (Tiangen Biotech Ltd., Beijing, China). They were then reverse-transcribed into cDNA using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA), according to manufacturer instructions.

Expression levels of *TNF-α, IL-1β, Intercellular adhesion molecule-1 (ICAM-1),* and *vascular cell adhesion molecule-1 (VCAM-1)* were semi-quantitatively detected using a StepOnePlus™

Table 1. Primer sequences used for RT qPCR

Target	Forward and Reverse Sequences
TNF-α	5'-AAATGGGCTCCCTCTCATCAGTTC-3'
	5'-TCTGCTTGGTGGTTTGCTACGAC-3'
IL-1β	5'-CAGCAATGGTCGGGACATAGTT-3'
	5'-GCATTAGGAATAGTGCAGCCATCT-3'
ICAM-1	5'-AGGTTTGCAGTAGTTGGGA-3'
	5'-GCGGGGTATATGGTGTCAGA-3'
VCAM-1	5'-TGTGCCTTGCGGATGGT-3'
	5'-GAAGTGTGCCCGAAATATGGA-3'
GADPH	5'-ACCACAGTCCATGCCATCAC-3'
	5'-TCCACCACCCTGTTGCTGTA-3'

Real-time PCR System (Applied Biosystems, Foster City, CA) with SYBR Green Super premix Taq II (TakaRa, Tokyo, Japan). Briefly, 2 µl of cDNA was used as the starting volume to amplify DNA. Next, 0.4 µl of the primer mix, 0.4 µl of ROX, 7.2 µl of RNase-free water, and 10 µl of SYBR were added to obtain a final volume of 20 µl. The PCR procedure was as follows: 95°C for 30 seconds; 40 cycles of 95°C for 5 seconds, 58°C for 30 seconds, and 72°C for 30 seconds; and 15 seconds for plate reading. A melting curve from 60°C to 95°C was obtained. Housekeeping gene *GADPH* was used as a control. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences for SYBR green probes of target genes are listed in **Table 1**.

Statistical analysis

All experiments were repeated at least 3 times. Results are presented as mean ± standard deviation (SD). Statistical analyses were implemented in SPSS (Version 19.0; IBM software, New York, USA). Multiple groups of comparisons were conducted with one-way analyses of variance (ANOVA), followed by LSD (L)/Dunnett's T3 post-hoc test where appropriate. $P<0.05$ indicates statistical significance.

Results

Etanercept administration ameliorated liver injuries induced by BD

Liver function was assessed by measurements of plasma AST and ALT. In the BD + saline group, serum ASL and ALT levels were significantly increased, compared with sham + saline rats, indicating worsen liver function (**Figure 1**, $P<0.01$). In contrast, in the BD + etanercept

group, there was a significant reduction in serum AST and ALT levels, compared with BD + saline rats ($P<0.05$). Although etanercept-treatment in sham rats also showed a trend of reduced AST and ALT levels, compared with sham + saline rats, neither of them reached statistical difference (**Figure 1**).

Furthermore, this study also assessed hepatic parenchymal injuries via histological observations. As shown in **Figure 2**, sham-operated rats with saline treatment had near normal liver architectures, while animals subjected to BD displayed obvious pathological changes, including distortion of liver architecture, narrow liver sinusoid, hepatocyte swelling, vascular congestion, fatty degeneration, and cytoplasm rarefaction. In contrast, etanercept treatment attenuated hepatocellular injuries, evidenced by reductions in pathological changes. No marked focal necrosis was observed in any of these groups.

Etanercept neutralized systemic TNF-α induced by BD

Levels of circulating TNF-α were evaluated by ELISA. Serum TNF-α levels were significantly elevated 6 hours after BD establishment, compared with those in sham + saline rats ($P<0.01$). Etanercept treatment, immediately after BD establishment, resulted in significantly lower levels of serum TNF-α, compared to BD + saline rats ($P<0.01$) (**Figure 3A**). Interestingly, sham operated treatment with etanercept also showed reduced serum TNF-α levels, compared with sham + saline rats ($P<0.01$).

To further evaluate whether BD induces expression of hepatic TNF-α and whether etanercept could also diminish levels of hepatic TNF-α, protein levels of TNF-α were assessed by IF analysis. As shown in **Figure 3B**, more TNF-α immunoreactivity was detected in BD + saline rats than in the other three groups. In contrast, TNF-α expression was markedly reduced in sections prepared from BD rats with etanercept administration, demonstrating that etanercept could effectively neutralize hepatic TNF-α.

Etanercept inhibited hepatic inflammation induced by BD

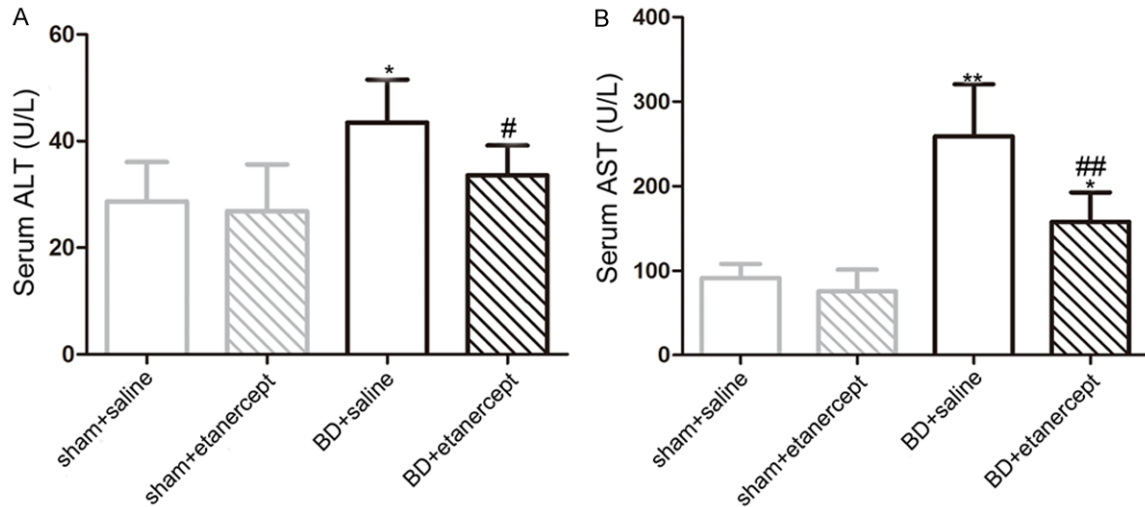To determine the effects of TNF-α blockage with etanercept on expression of hepatic

Figure 1. Etanercept attenuated BD-induced hepatic injury. Serum levels of ALT (A) and AST (B) were measured 6 hours after the induction of BD. Data are expressed as mean ± SD (n=8 per group). *$P<0.05$ versus the sham + saline group. **$P<0.01$ versus the sham + saline group. #$P<0.05$ versus BD group. ##$P<0.01$ versus the sham + saline group.
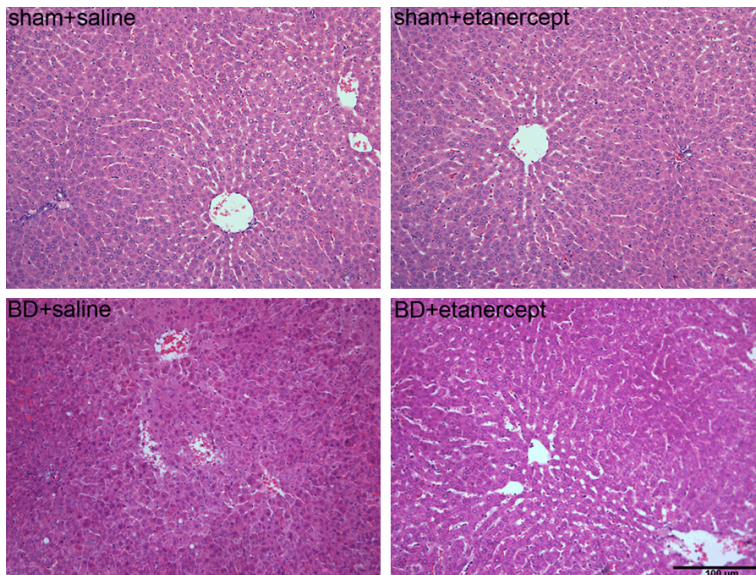

Figure 2. Hepatic histological changes in each group. Six hours after BD establishment, rats (n=8 per group) were sacrificed and liver sections were subjected to H&E staining (Bar=100 μm).

inflammatory molecules in BD rats, mRNA levels of *TNF-α, IL-1β,* and adhesion molecules (*VCAM-1* and *ICAM-1*) in hepatic tissues were assessed. These inflammatory genes were remarkably upregulated following BD ($P<0.05$ versus sham + saline group). However, etanercept administration significantly reduced expression of these genes to lower levels, compared with rats in the BD group ($P<0.05$) (**Figure 4**).

To further quantify inflammatory cell infiltration in liver tissues, this study analyzed liver sections by immunohistochemistry for neutrophils (MPO staining). As shown in **Figure 5**, quantification revealed significantly a higher number of neutrophils in liver tissues from BD + saline rats, compared with sham + saline rats ($P<0.05$). Although etanercept treatment, immediately after BD, resulted in reduced neutrophil numbers in liver, this decline did not reach statistical significance.

Etanercept inhibited hepatic apoptosis induced by BD

Many studies have indicated that BD results in hepatic apoptosis and that TNF-α plays a vital role in hepatic apoptosis. Accordingly, the current study examined whether treatment with etanercept could block hepatic apoptosis in a BD rat model. In this study, liver apoptosis was confirmed quantitatively by a TUNEL assay and *in-situ* IF analysis of caspase-3 activation. In hepatic tissues from sham-operated rats, a very low level of TUNEL-positive staining could be observed. In contrast, the rate of TUNEL-
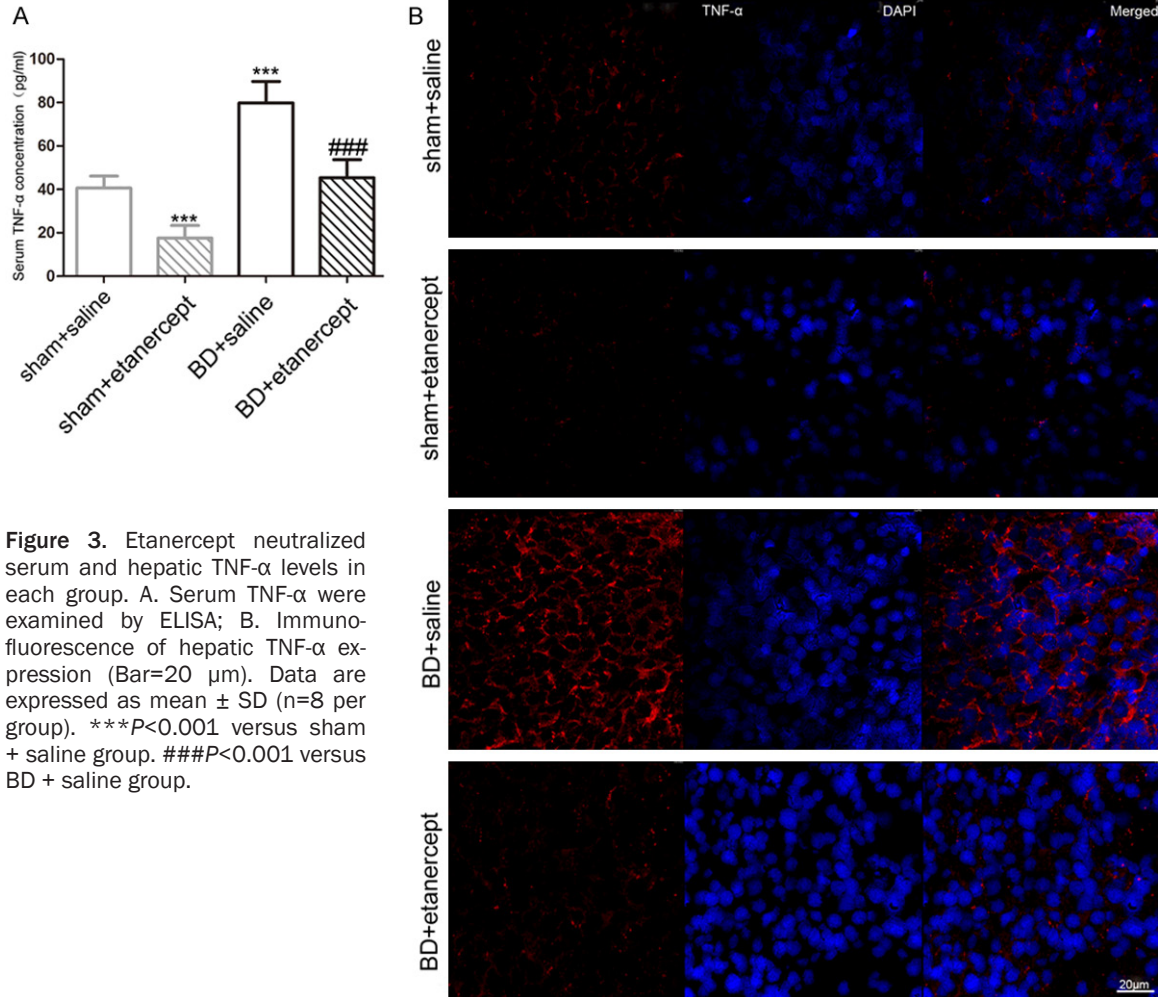
Figure 3. Etanercept neutralized serum and hepatic TNF-α levels in each group. A. Serum TNF-α were examined by ELISA; B. Immunofluorescence of hepatic TNF-α expression (Bar=20 μm). Data are expressed as mean ± SD (n=8 per group). ***$P<0.001$ versus sham + saline group. ###$P<0.001$ versus BD + saline group.

positive cells was significantly increased in hepatic tissues derived from BD rats. Administration of etanercept, immediately after BD establishment, showed remarkable anti-apoptotic effects, evidenced by a reduction in TUNEL-positive staining ($P<0.01$ versus BD + saline group) (**Figure 6**). This study also examined cleaved caspase-3 by IF, due to its critical role in the course of TNF-α-mediated apoptosis [20]. Consistent with the results of TUNEL assay, BD rats demonstrated notably increased hepatic caspase-3 activation, relative to sham + saline rats (3.68±1.54 versus 14.57±2.68% cleaved caspase-3 positive cells; $P<0.05$). Rats treated with etanercept, immediately after BD establishment, had remarkably diminished hepatic caspase-3 cleavages, compared with BD + saline rats (9.32±3.10 versus 14.57± 2.68% cleaved caspase-3 positive cells; $P<0.01$) (**Figure 7**).

Discussion

The primary purpose of this study was to determine whether etanercept, a TNF-α inhibitor, could protect the liver from BD-induced injuries in a rat model. Results showed that etanercept treatment, immediately after BD establishment, significantly attenuated BD-induced hepatic injury, evidenced by diminished levels of serum aminotransferases and histopathological improvement. Mechanisms underlying these effects may be related to the inhibition of hepatic inflammation and apoptosis.

BD, defined as an irreversible injury of the cerebrum, cerebellum, and brain stem, is associated with various systemic physiological changes, including hemodynamic changes, hormonal changes, and inflammatory response [21]. BD may cause progressive deterioration of donor organs, including the liver, leading to a lower

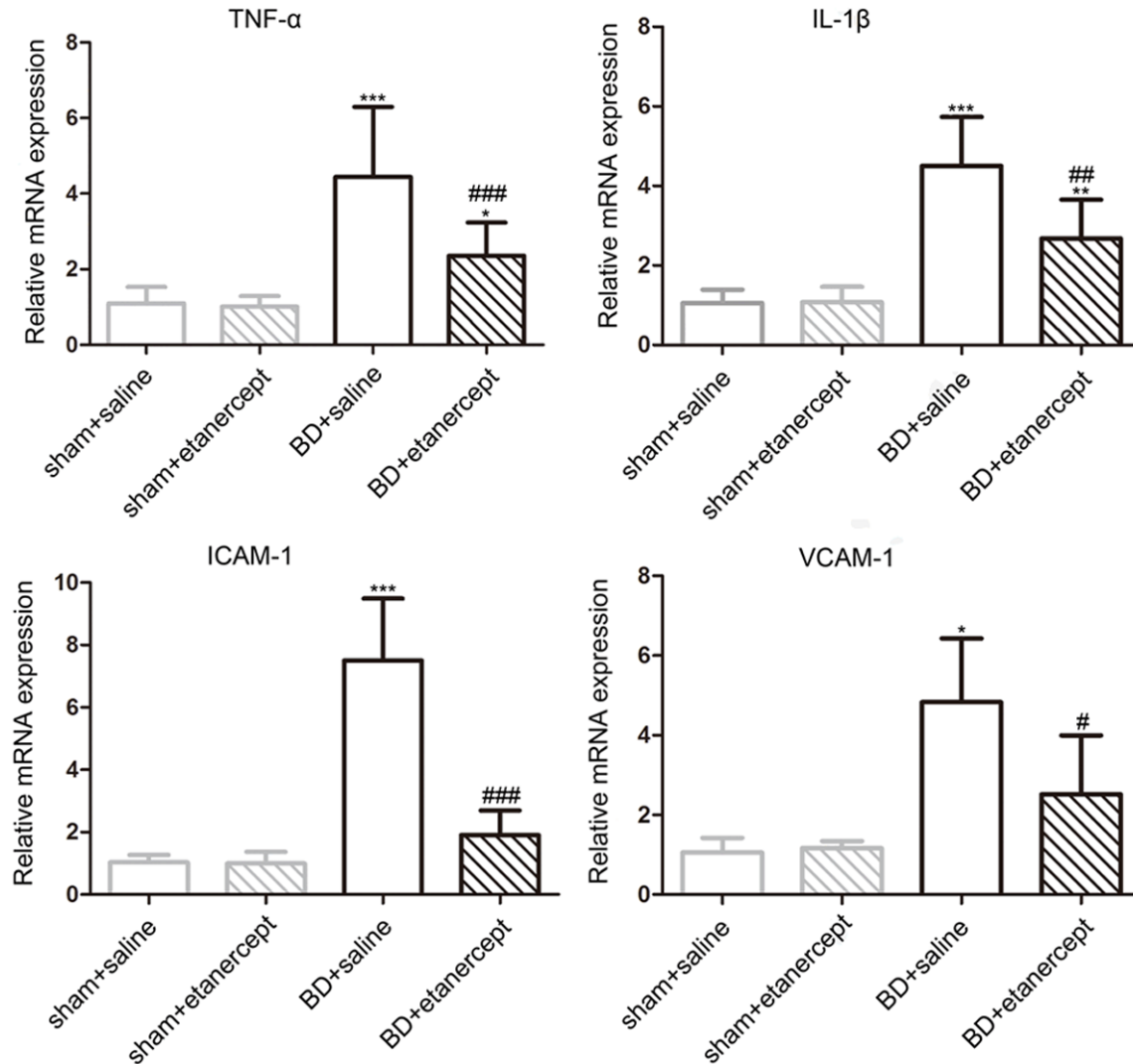**Figure 4.** Etanercept reduced mRNA expression of *TNF-α, IL-1β, ICAM-1, and VCAM-1* in the liver tissue. Data are expressed as mean ± SD (n=8 per group). *P<0.01 versus the sham + saline group. *P<0.001 versus sham + saline group. ***P<0.001 versus sham + saline group. #P<0.05 versus BD + saline group. ##P<0.01 versus BD + saline group. ###P<0.001 versus BD + saline group.

graft survival after transplantation, compared with organs from living donors [2, 21]. AST and ALT are well-known markers of hepatocyte damage. Previous studies have demonstrated that serum ALT and AST levels are increased after BD [2, 22]. In the present study, levels of AST and ALT were significantly higher in BD + saline rats than in the sham + saline group, indicating that BD did cause liver damage in rats. Furthermore, histological observations demonstrated that BD induced hepatic parenchymal injuries, evidenced by deteriorative histopathological changes in liver tissues from BD + saline rats.

The systematic inflammatory response elicited by BD has been thought to be a causal mechanism for these injuries. TNF-α is the main inflammation mediator after BD [23]. Elevated levels of TNF-α in systemic circulation, as well as in tissues, have been observed in both humans and animal brain-dead donors [5, 23, 24]. In the present study, levels of circulating TNF-α were significantly increased in rats subjected to BD, compared with sham + saline rats. This study also detected TNF-α expression in liver tissues by *in situ* IF, observing a greater number of TNF-α-positive cells in the livers of BD rats, compared to sham + saline rats. These
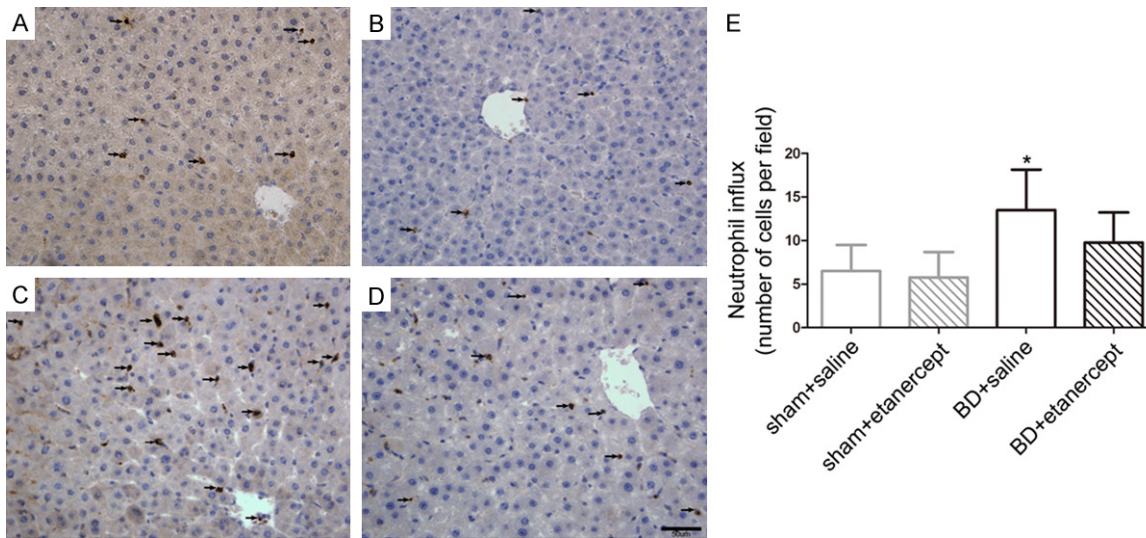
Figure 5. Etanercept mitigated BD-induced hepatic neutrophil infiltration. A-D. Show liver tissues from sham + saline, sham + etanercept, BD + saline, and BD + etanercept rats, respectively; E. Neutrophil infiltration quantification in the liver, shown as the number of positive cells was performed by two researchers in 10 adjacent non-overlapping visual fields at a magnification of ×400 (Bar=50 µm). Data are expressed as mean ± SD (n=8 per group). *$P<0.05$ versus sham + saline animals.

observations were in line with the results by Hoeger et al. [25], in which they evaluated serum TNF-α concentrations and mRNA levels of hepatic TNF-α in rats. They found that TNF-α concentrations, as well as mRNA levels, of hepatic TNF-α were increased in BD rats, compared with sham rats.

TNF-α, a primary mediator of systemic inflammation, is a cytokine that stimulates acute inflammation. It likely plays a role in the initiation of the inflammatory cascade. TNF-α is secreted by various types of cells, such as macrophages, cerebral microglia, astrocytes, and hepatic Kupffer cells. In a brain-dead state, circulating TNF-α is mainly produced by cerebral microglia and astrocytes [26]. Under severe cerebral injuries, cerebral microglia and astrocytes are stimulated and produce cytokines, including TNF-α [27]. These cytokines cross the blood-brain barrier and reach peripheral organs and tissues, further increasing expression of TNF-α and other cytokines. In addition to the release of injured cerebrum, hormonal and metabolic derangement, catecholamine storm, and impairment of the parasympathetic nervous system, BD may also cause the upregulation of TNF-α [4, 25]. Recently, Campbell et al. [16] showed that TNF-α is also an acute-phase protein produced by hepatic Kupffer cells in response to brain injuries.

TNF-α not only induces its own secretion, but also has the ability to increase the release of other inflammatory cytokines, including IL-1β, induce expression of cell adhesion molecules, such as ICAM-1 and VCAM-1, and influence neutrophil recruitment [28]. Therefore, inhibition of TNF-α will suppress the production of inflammatory cytokines and reduce expression of cell adhesion molecules. Systemic treatment with etanercept has been documented to be effective in inhibiting TNF-α in various animal models of liver injury, resulting in improved outcomes [18, 19]. In the current study, etanercept administration successfully neutralized increased TNF-α in serum and hepatic tissues 6 hours after BD. As expected, TNF-α blockade with etanercept significantly reduced the upregulated mRNA expression levels of *TNF-α, IL-1β, VCAM-1,* and *ICAM-1* in the livers after BD. As with TNF-α, IL-1β is also an important pro-inflammatory cytokine. It not only causes inflammation itself, but more importantly induces expression of many other pro-inflammatory cytokines (TNF-α) and adhesion molecules [29, 30]. IL-1β also induces endothelial activation, promoting leukocyte interactions with adhesion molecules expressed on the cell surface [31]. VCAM-1 and ICAM-1 are two important adhesion molecules that facilitate leukocyte adhesion to the sinusoids [32]. Thus, TNF-α block-

Apoptosis is another mechanism in liver injuries. Liver apoptosis after BD has previously been identified in many animal models. TNF-α has been shown to be a determinant of BD-induced liver apoptosis [5]. Previous studies have indicated that TNF-α induces apoptotic cell death by activating signaling pathways, such as NF-κB, c-Jun N-terminal kinase, and p38-mitogen-activated protein kinase pathways [33]. Thus, it was hypothesized that anti-TNF-α treatment will ameliorate liver apoptosis induced by BD. As expected, etanercept treatment effectively attenuated TUNEL-positive hepatocytes, while massive hepatic apoptosis was detected in BD rats. Activation of caspase-3-like proteinase is essential for TNF-α-induced hepatic apoptosis [20]. Rebolledo et al. [34] found that significantly increased hepatic mRNA expression of TNF-α was accompanied by significantly increased expression of caspase-3 in slow-inducted BD rats, compared with fast-inducted BD rats. In this study, consistent with the results of the TUNEL assay, etanercept treatment decreased cleaved caspase-3 expression in liver tissues after BD. Therefore, current results suggest that etanercept administration, immediately after BD, may alleviate BD-induced liver apoptosis, probably via the neutralization of hepatic apoptosis provoked by TNF-α.

Figure 6. Etanercept decreased hepatic apoptosis induced by BD. Analyses performed following 6 hours of BD in the BD groups and 6 hours of ventilation in the sham groups. A-D. Represent TUNEL staining at a magnification of ×400 from sham + saline, sham + etanercept, BD + saline, and BD + etanercept rats, respectively. Black arrows show examples of TUNEL positive cells. Black arrows show examples of positive cells. Bar represents 50 µm; E. Quantification of TUNEL positive cells, shown as percentage of positive cells was performed by two researchers in 10 adjacent non-overlapping visual fields at a magnification of ×400. Data are expressed as mean ± SD (n=8 per group). ***P<0.001 versus sham + saline animals. ##P<0.01 versus BD + saline group.

ade with etanercept finally reduces the neutrophil migration and infiltration into liver tissues via suppressing the production of itself and the abovementioned inflammatory cytokines. In fact, current results demonstrated that inhibition of TNF-α with etanercept, immediately after BD, suppressed liver infiltration of neutrophils.

As a result, administration of etanercept, immediately after BD establishment, significantly decreased liver aminotransferases. Consequently, current results indicate that inhibition of TNF-α could ameliorate BD-induced liver injuries. Consistent with improved liver damage parameters, etanercept-treated animals showed improved histopathological changes, includ-
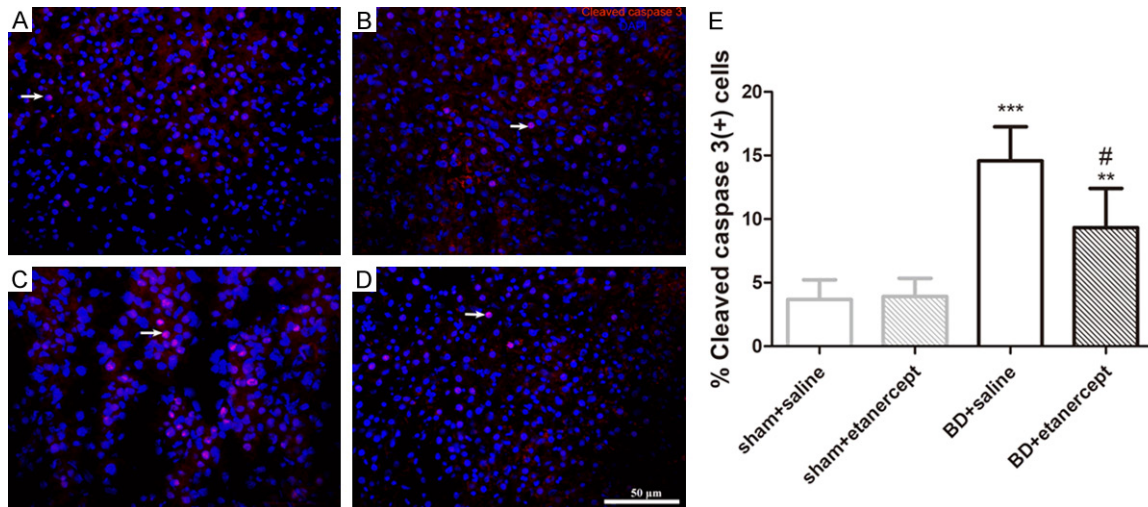
Figure 7. Etanercept reduced hepatic cleaved caspase-3 expression induced by BD. IF was performed on liver sections after 6 hours of BD or sham operation. A-D. Show liver tissues from sham + saline, sham + etanercept, BD + saline, and BD + etanercept rats, respectively. White arrows show examples of positive cells. Bar represents 50 μm; E. Quantification of cleaved caspase-3 positive cells, shown as percentage of positive cells was performed by two researchers in 10 adjacent non-overlapping visual fields at a magnification of ×400. Data are expressed as mean ± SD (n=8 per group). ***$P<0.001$ versus sham + saline animals. ##$P<0.01$ versus BD + saline group.

ing a well-preserved hepatic architecture, compared with the histopathological findings for BD + saline rats. Therefore, present data suggests that etanercept protects against BD-induced liver injuries through the amelioration of inflammation and apoptosis.

In summary, present findings demonstrate that the administration of etanercept, immediately after BD, in rats attenuates BD-induced liver injuries. Additionally, the protective effects of etanercept are closely associated with inhibition of the inflammatory response and apoptosis. Current findings suggest that inhibition of TNF-α is a potential protective strategy for the liver transplantation in brain-dead donor management, before organ procurement.

Disclosure of conflict of interest

None.

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