European Review for Medical and Pharmacological Sciences 2014; 18: 10-27

Effect of etanercept and lithium chloride on preventing secondary tissue damage in rats with experimental diffuse severe brain injury

M.A. EKICI[1], O. UYSAL[2], H.I. CIKRIKLAR[3], Z. ÖZBEK[4], D. TURGUT COSAN[5], C. BAYDEMIR[6], B. KAZANCI[7], D. HAFIZOĞLU[8]

[1]Neurosurgery Clinic, Ministry of Health, Sevket Yilmaz Research and Training Hospital, Bursa, Turkey
[2]Vocational School of Health Services, Eskisehir Osmangazi University, Eskisehir, Turkey
[3]Emergency Clinic, Ministry of Health, Sevket Yilmaz Research and Training Hospital, Bursa, Turkey
[4]Department of Neurosurgery, Eskisehir Osmangazi University, Medical Faculty, Eskisehir, Turkey
[5]Department of Medical Biology, Eskisehir Osmangazi University, Medical Faculty, Eskisehir, Turkey
[6]Department of Biostatistics, Eskisehir Osmangazi University, Medical Faculty, Eskisehir, Turkey
[7]Department of Neurosurgery, Ufuk University, Medical Faculty, Ankara, Turkey
[8]Department of Pediatric Immunology, Uludag University, Medical Faculty, Bursa, Turkey

Abstract. – OBJECTIVE: Studies in animals have provided key evidence that antagonizing TNF-α is a viable therapeutic strategy for diffuse severe brain injury. This study is planned to prevent post-traumatic secondary tissue damages in rat diffuse severe brain injury model, which is induced by alone or combined administration of Etanercept and lithium chloride (LiCl).

MATERIALS AND METHODS: Male Sprague-Dawley rats were used in the current study. Rats were divided into 5 groups. Trauma was not induced and treatment was not applied to rats of Sham group. For rats of Trauma+Saline group, saline 0.9% was administered via intraperitoneal (i.p.) route at dose of 1 mg/100 g body weight 1 hour after trauma. For rats of Trauma+Etanercept group, Etanercept was administered via i.p. route at dose of 5 mg/kg body weight 1 hour after trauma. For rats of Trauma+LiCl group, LiCl was administered via i.p. route at dose of 50 mg/kg body weight 1 hour after trauma. For rats of Etanercept+LiCl group, Etanercept and LiCl were administered via i.p. route at dose of 5 mg/kg body weight and 50 mg/kg body weight, respectively, 1 hour after trauma. Serum glial fibrillary acidic protein (GFAP) and Tau levels were analyzed with ELISA. For analyses H&E, TUNEL, GFAP and TNF-α staining methods were used.

RESULTS: We demonstrate that Etanercept treatment reduced the TBI-induced brain tissues alteration, reduced the expression of TNF-α and improve edema and axonal swelling. We observed a significant decrease in TNF-α and GFAP positivity after LiCl was administered.

CONCLUSIONS: The findings obtained in this study suggest that the combination therapy with Etanercept and LiCl decreased neuronal degeneration and alleviated secondary tissue damage in post-traumatic period.

Key Words:
Traumatic brain injury, Etanercept, Lithium chloride, TNF-α.

Introduction

Traumatic brain injury (TBI), the leading cause of morbidity and mortality in young adults and children, is a major public health problem globally[1-5]. Depending on severity and duration, brain injury may cause either infarction or selective neuronal death[6].

Injury to the cerebral vasculature breaks the blood-brain barrier (BBB) is accompanied by an inflammatory response marked by infiltration of neutrophils and macrophages, activation of glial cells and up-regulated expression of pro-inflammatory cytokines[7-9]. The molecular events result in apoptosis, reactive astrogliosis, microglial activation, infiltration of immune cells in the central nervous system (CNS), neurodegeneration, altered plasticity and neuronal regeneration[4,10-12].

Diffuse brain injury includes diffuse axonal injury (DAI), diffuse vascular injury and diffuse hypoxia/ischaemia. One of the major pathological outcomes of these mechanisms is DAI, the main clinical feature of human TBI, leading to diffuse degeneration of cerebral white matter[13,14]. Tau protein is a microtubule-associated protein

Corresponding Author: Onur Uysal, Ph.D; e-mail: ouysal1971@gmail.com

that is primarily localized in the axonal compartment of neurons. Loss of axonal microtubules following injury releases intracellular microtubule binding proteins, such as Tau, into the extracellular space, where they are transported to the cerebral spinal fluid (CSF). CSF levels of a cleaved form of Tau (C-tau) reflect axonal damage after head injury, Alzheimer's disease and meningitis[5,15,16].

Astrocytes are the most numerous non-neuronal cell type in the CNS and make up about 50% of human brain volume. Within a few hours of virtually any type of brain injury, surviving astrocytes in the affected region begin to exhibit hypertrophy and proliferation, termed "reactive astrogliosis". This response is fortified by the migration of microglia and macrophages to the damaged area. Reactive astrocytes increase the expression of their structural protein, Glial fibrillary acidic protein (GFAP), which is commonly used as astrocyte markers[2,6,17,18]. Increasing numbers of GFAP(+) astroglial cells following TBI have been described in several experimental studies in animals[19,20]. Astrocytes are known to be a source of pro-inflammatory cytokines and in the advanced stages of injury progression form a glial scar inhibitory to neural regeneration. These observations suggest that conversion of glial cells to their "reactive" state, and the associated increase in expression of cytokines and chemokines, may play a role in neurodegeneration[3].

TNF-α is 17 Kda major pro-inflammatory cytokine involved with development of cerebral edema and secondary neuronal loss after TBI. TNF-α may contribute to ischemic brain damage through a variety of pro-inflammatory effects, such as alteration of BBB permeability, activation of microglia and astrocytes, induction of cellular adhesion molecule expression and recruitment of neutrophils[21,22]. Clinically, elevated levels of TNF-α has been detected in CSF and serum from patients with head trauma[1,23].

Etanercept is a TNF-α antagonist that represents a dimeric fusion protein of the extracellular ligand-binding portion of the soluble 75-kDa TNF-α receptor II (p75-TNFR, or TNFRII) and the fragment-crystallizable (Fc) portion of human immunoglobulin (IgG)[24-27]. More recent studies showed that Etanercept was able to intervene in the brain inflammatory response and to protect brain and spinal cord from secondary damage caused by infiltrating leukocytes. This raised the possibility that experimental TBI could be affected by Etanercept therapy[1,28].

Lithium has now been shown to be neuroprotective in animal models of stroke, Huntington's disease, and other neurodegenerative diseases[29]. Su et al[30] reported that Lithium can increase the proliferation and neuronal differentiation of spinal cord-derived neural progenitor cells (NPCs) after transplantation into the intact spinal cord of adult rats, indicating that Lithium may have a therapeutic potential in cell transplantation strategies to treat neurological disorders. However, the precise mechanisms of how Lithium exerts its promoting effects on the proliferation and neuronal generation of NPCs *in vitro* and *in vivo* remain unknown.

This study is planned to prevent post-traumatic secondary tissue damages in rat diffuse severe brain injury model, which is induced by alone or combined administration of Etanercept and LiCl (lithium chloride).

Materials and Methods

Animals

In this study Male Sprague-Dawley rats (n=40) weighing 250 ± 50 g were obtained from the Eskisehir Osmangazi University Experimental Research Center. They were used after 2 weeks of adaptation. The rats were housed in polycarbonate cages in a temperature (21 ± 1^0C) and humiditiy (45-55%) controlled room which was maintained on a 12/12 reversed light-dark cycle and were fed with a standard rat chow (Oguzlar Yem, Eskisehir, Turkey) and allowed to drink *ad libitum*. The procedures involving animal care, surgery and sample preparation were approved by the Institutional Animal Care and Experiments Committee of Eskisehir Osmangazi University Faculty of Medicine, and the Regulations and Guidelines for the Care and Use of Laboratory Animals of Institutional Animal Care and Experiments Committee of Eskisehir Osmangazi University Faculty of Medicine (23.02.2012/45-262), and the Guidelines laid down by the NIH in the US regarding the care and use of animals for experimental procedures were observed.

Experimental Groups

In this experimental study, experimental diffuse severe head injury was examined in five groups (n=8). Animals were sedatized with intramuscular (i.m.) xylazine 10 mg/kg (Rompun, Bayer Ilaç San, Istanbul) and anesthesia was in-

duced with i.m. ketamine hydrochloride 50 mg/kg (Ketalar, with licence Parke-Davis lisansi ile Eczacibasi Ilaç San, Istanbul).

Trauma was not induced and treatment was not applied to rats of Sham group.

For rats of Trauma+Saline group, saline 0.9% was administered via intraperitoneal (i.p.) route at dose of 1 mg/100 g body weight 1 hour after trauma.

For rats of Trauma+Etanercept group, Etanercept was administered via i.p. route at dose of 5 mg/kg body weight 1 hour after trauma.

For rats of Trauma+LiCl group, LiCl was administered via i.p. route at dose of 50 mg/kg body weight 1 hour after trauma.

For rats of Etanercept+LiCl group, Etanercept and LiCl were administered via i.p. route at dose of 5 mg/kg body weight and 50 mg/kg body weight, respectively, 1 hour after trauma.

Blood was drawn and cerebral tissue specimens were biopsied for biochemical and histological analyses, respectively, at 24 hours following the induction of trauma, and all animals were sacrificed.

Induction of Head Trauma

Diffuse severe head injury was induced using the modified version of the model which was described by Marmarou et al[31]. Briefly, after anesthesia was induced, rats of all groups were placed prone position; scalp was shaved and disinfected with betadine. Following vertical skin incision, periosteum was dissected. The trauma device consists of a column of brass weights falling freely by gravity onto a metallic helmet fixed to the skull vertex of the rat by dental acrylic. The brass weights were threaded so that they could be connected to produce a falling weight 100 g. From a designated height, the weight falls through 100 cm vertical section of a transparent Plexiglas tube held in place with a ring stand. The helmet is a stainless-steel disc 10 mm in diameter and 3 mm thick. The contact side of the disc is grooved concentrically to accept acrylic and firm the contact. The animal was placed in a prone position on a foam bed of known spring constant contained within a Plexiglas frame and secured in place with two belts. The lower end of the Plexiglas tube was then positioned directly above the helmet. Diffuse severe brain injury was delivered by dropping the weight from a predetermined height. Rebound impact was prevented simply by sliding the Plexiglas box (foam bed) containing the animal away from the tube immediately following the initial impact. The rats with post-traumatic fracture of skull or died secondary to trauma were excluded.

Detection of GFAP Levels

Rat GFAP levels were detected in the serum by Rat GFAP ELISA kit (Eastbiopharm, Hangzhou). Firstly samples and standards were added into the well. Antibodies were labeled with enzyme and the plate was incubated 60 minutes at 37ºC. Then plate was washed five times and chromogen solutions were added. It was incubated 10 minutes at 37ºC and stop solution added into wells. Optical density (OD) was measured under 450 nm wavelengths with a microplate reader (LabSystems, UV/Vis. Spectrum Finstruments™ Multiskan Model 347 Finland). According to standards concentration and the corresponding OD values were calculated out the standard curve linear regression equation to calculate the corresponding sample's concentration.

Detection of Tau Protein Levels

The protein levels rat Tau protein in the serum were quantified using Enzyme Linked Immunosorbent Assay Kit (ELISA) for microtubule associated protein Tau (Uscn, PR China). Samples, standards and blank were added into the appropriate wells. Then detection reagents were added and plate was incubated 60 minutes at 37ºC. Plate was washed five times. Substrate solution was added to each well and it was incubated 20 minutes at 37ºC. Finally stop solution was added into the well. Measurement was conducted using ELISA reader (LabSystems, UV/Vis. Spectrum Finstruments™ Multiskan Model 347 Finland) at 450 nm.

Histological Examinations

H&E Staining

Brain tissue was carefully excited, fixed in neutral buffered formalin for histologic analyses. After the fixation, the tissues were embedded in paraffin and serial sections (4 μm) were prepared for each of the paraffin blocks. On average, 50 sections were collected per rat. Sections were stained for histological analyses with Hematoxylin and Eosin (H&E). Digital images were obtained by an Olympus BX-61 (Olympus America Inc., Hauppauge, NY, USA) microscope with a DP70 digital camera. The histological evaluation of the brain injury was semi-quantitatively scored by two independent observers

who undertook the evaluation using an ocular micrometer by light microscopy. The severity of the histopathologic changes and neuronal degenerative changes was scored viz. (0), no injury; (1), mild; (2), moderate and (3) dense/common. The examination of the histologic changes consisted of the evaluation of the following: vascular congestion, intraparenchymal hemorrhage, inflammation, edema and gliosis. The examination of the neuronal degenerative changes consisted of the evaluation of the following: nuclear pyknosis, nuclear hyperchromasia, cytoplasmic eosinophilia, and axonal swelling[18,32-38].

In situ Apoptosis Detection

Cleavage of genomic DNA during apoptosis may yield double-stranded, low molecular-weight DNA strand breaks that can be labeled by terminal deoxynucleotidyl transferase (TdT) which catalyzes polymerization of labeled nucleotides to free 3'-OH ends in an template-independent manner (Terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labeling, TUNEL reaction). The quantity of apoptotic cell death was evaluated using an *in situ* cell death detection kit (Cat. no.S7101; Chemicon International-ApopTag Plus Peroxidase Kits, Temecula, CA, USA). Firstly, the serial sections were deparaffinized three times in xylene. The sections were then rehydrated through a series of decreasing concentrations of ethanol before the slides were washed in phosphate-buffered saline (PBS). After that, the sections were subjected to partial digestion with proteinase K (20 μg/ml) (Merck) at room temperature for 15 min and were washed 2 times in PBS. Thereafter, the sections were incubated at room temperature in an equilibration buffer. The tissue sections were incubated at 37°C for 60 min with the TUNEL reaction mixture (70% reaction buffer, 30% TdT enzyme) in a humidified chamber in the dark. The slides were agitated and incubated in the stop/wash buffer for 10 min at room temperature. After the incubation, the slides were washed 3 times with PBS (phosphate buffered saline). Then, anti-digoxignenin conjugate was applied to the slides and incubated in a humidified chamber for 30 min at room temperature. The slides were washed 4 times with PBS. 3-3 diaminobenzidine (DAB) was then added onto the slides for 10 min, and the slides were washed in distilled water three times before drying. For analysis of TUNEL(+) motor neurons located at perilesional region of the cortex, two independent observers evaluated 25 optical fields for each 50 sections per animal chosen randomly using a x40 ocular micrometer by light microscopy and median 25%-75% percentiles were calculated[35,37,39].

GFAP Immunohistochemistry (Avidin-biotin method)

To identify protoplasmic astrocytosis, the GFAP immunohistochemical staining was performed on formalin-fixed, paraffin-embedded brain tissue. Three sections from each brain were examined. In each case, 4 μm thick serial paraffin sections were obtained on poly-L-lysine coated slides. The sections were dewaxed, put in 0.3% H_2O_2 in methanol for 30 min for antigen retrieval, and incubated with 1% horse serum and 1% bovine serum albumin (BSA) mixture for 30 min to reduce nonspecific binding. Monoclonal mouse anti-human primary antibody to GFAP (Novocastra Laboratories, Ltd., Newcastle-upon-Tyne, UK), was applied at a concentration of 1:200 and incubated at room temperature for 90 min. After three washes in 0.01 M PBS for 10 min each, biotinylated horse anti-mouse secondary antibody (1:100, Vectastain Elite ABC kit; Vector Laboratories, Inc., Burlingame, CA, USA) was applied for 30 min and followed by avidin-biotin complex for another 30 min. DAB (DAB kit; Vector Laboratories, Inc.) was used for substrate chromogen staining for 2 to 5 min. The sections were counterstained with 0.1% Mayer's hematoxylin (Sigma Diagnostics, St. Louis, MO, USA) and mounted. For analysis of GFAP(+) protoplasmic astrocytes located at perilesional region of the cortex, two independent observers evaluated 25 optical fields for each 50 sections per animal chosen randomly using a x40 ocular micrometer by light microscopy and median 25%-75% percentiles were calculated[18,33-35,38,40-43].

TNF-α Immunohistochemistry

The TNF-α immunohistochemical staining was performed on formalin-fixed, paraffin-embedded brain tissue. Three sections from each brain were examined. In each case, 4 μm thick serial paraffin sections were obtained on Poly-L-Lysine coated slides and deparaffinized in histoclear, rehydrated through graded ethanol, treated with antigen unmasking solution and 0.3% H_2O_2 at room temperature for 20 min. Sections were blocked with Clean Vision™ blocking solution (Immuno Vision Technologies Co, Hillsborough, CA, USA) at room temperature for 2 h and then incubated overnight at 4°C with goat polyclonal

anti-TNF-α antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:200). After washes in 0.05 M PBS, sections were incubated with biotinylated donkey anti-goat (1:200) for 1 h, and avidin-biotin complex (Vector Laboratories Inc., Burlingame, CA, USA) for 1 h. Immunoreactivity was detected with 0.05% DAB and 0.03% H_2O_2. As negative controls, alternate sections were processed in parallel without primary antibody. The specificity of TNF-α antibody was verified using the relative blocking peptides (Santa Cruz Biotechnology) according to the manufacturer's instruction. Simply, anti-TNF-α 1 μg/ml combined with TNF-α blocking peptides 5 μg/ml were incubated at room temperature for 2 h. Following blocking, the antibody/peptide mixture was applied for immunostaining to verify the specificity of TNF-α antibodies.

Neuronal immunoreactivity was analyzed by counting 5 fields per sections from 4-6 sections for each subject Because of less frequent and more heterogenous immunoreactivity in neuronal, glial, and endothelial cells, their TNF-α expression was graded as follows: (0), no immunoreactivity; (1), trace of immunoreactivity, and (2) increased TNF-α immunoreactivity[44].

Statistical Analysis

SPSS version 15.0 (SPSS for Windows Inc., Chicago, IL, USA) was used for statistical analysis. Normal distribution of the data was evaluated with Kolmogorov-Smirnov test. Kruskal Wallis and ANOVA Tests with Tukey Multiple Comparison Tests and Holm Sidak Multiple Comparison Tests were used to compare the groups. The results were expressed as mean ± standard deviation and Median (25-75% percentiles). $p < 0.05$ was considered statistically significant.

Results

Serum GFAP and Tau Levels

Mean values of venous blood GFAP concentration was 0.292 ± 0.0752 for Sham group, 1.042 ± 0.561 for Trauma+Saline group, 0.795 ± 0.165 for Trauma+Etanercept group, 0.961 ± 0.222 for Trauma+LiCl group, 0.856 ± 0.179 for Trauma+Etanercept+LiCl group. Mean values of venous blood GFAP concentration was statistically significantly between groups ($p < 0.001$) (Table I).

Median (25%-75% percentiles) values of venous blood TAU concentration was 0.00550 (0.00385-0.0152) for Sham group, 0.0533 (0.0388-0.0650) for Trauma+Saline group, 0.0232 (0.0145-0.0462) for Trauma+Etanercept group, 0.0392 (0.0230-0.0447) for Trauma+LiCl group, 0.0439 (0.0240-0.0690) for Trauma+Etanercept/LiCl group. Median (25%-75% percentiles) values of venous blood GFAP concentration was statistically significantly between groups ($p = 0.009$) (Table II).

Histological Results

Etanercept Decreased Edema Axonal Swelling During TBI

Based on H&E staining, histopathological changes were examined on the perilesional region of the cerebral cortex, and a statistically significant difference was observed between study groups in comparison with Trauma+Saline group with respect to the vascular congestion, intraparenchymal hemorrhage, inflammation and gliosis. Etanercept remarkably suppressed edema in post-traumatic period ($p < 0.001$). This finding

Table I. Assessment of serum GFAP concentration (ng/ml) (Mean ± St. Deviation) for all groups.

Groups	Serum GFAP	F and *p*
Sham	0.292 ± 0.0752	
Trauma+Saline	1.042 ± 0.5610	
Trauma+Etanercept	0.795 ± 0.1650	8.038 $p < 0.001^{A,B,C,D}$
Trauma+LiCl	0.961 ± 0.2220	
Trauma+Etanercept +LiCl	0.856 ± 0.1790	

The statistical difference resulting from the comparison of the Trauma+Saline vs. Sham group (A), Trauma+LiCl vs. Sham group (B), Trauma+Etanercept+LiCl vs. Sham group (C) and Trauma+Etanercept vs. Sham group (D).

Table II. Assessment of median serum TAU concentrations (ng/ml; 25%-75% percentiles) for all groups.

Groups	Serum TAU	F and *p*
Sham	0.0055 (0.0038-0.0152)	
Trauma+Saline	0.0533 (0.0388-0.0650)	
Trauma+ Etanercept	0.0232 (0.0145-0.0462)	13.451 $p =$ 0.009 A
Trauma+LiCl	0.0392 (0.0230-0.0447)	
Trauma+ Etanercept+LiCl	0.0439 (0.0240-0.0690)	

The statistical difference resulting from the comparison of the Trauma+Saline vs. Sham group (A)

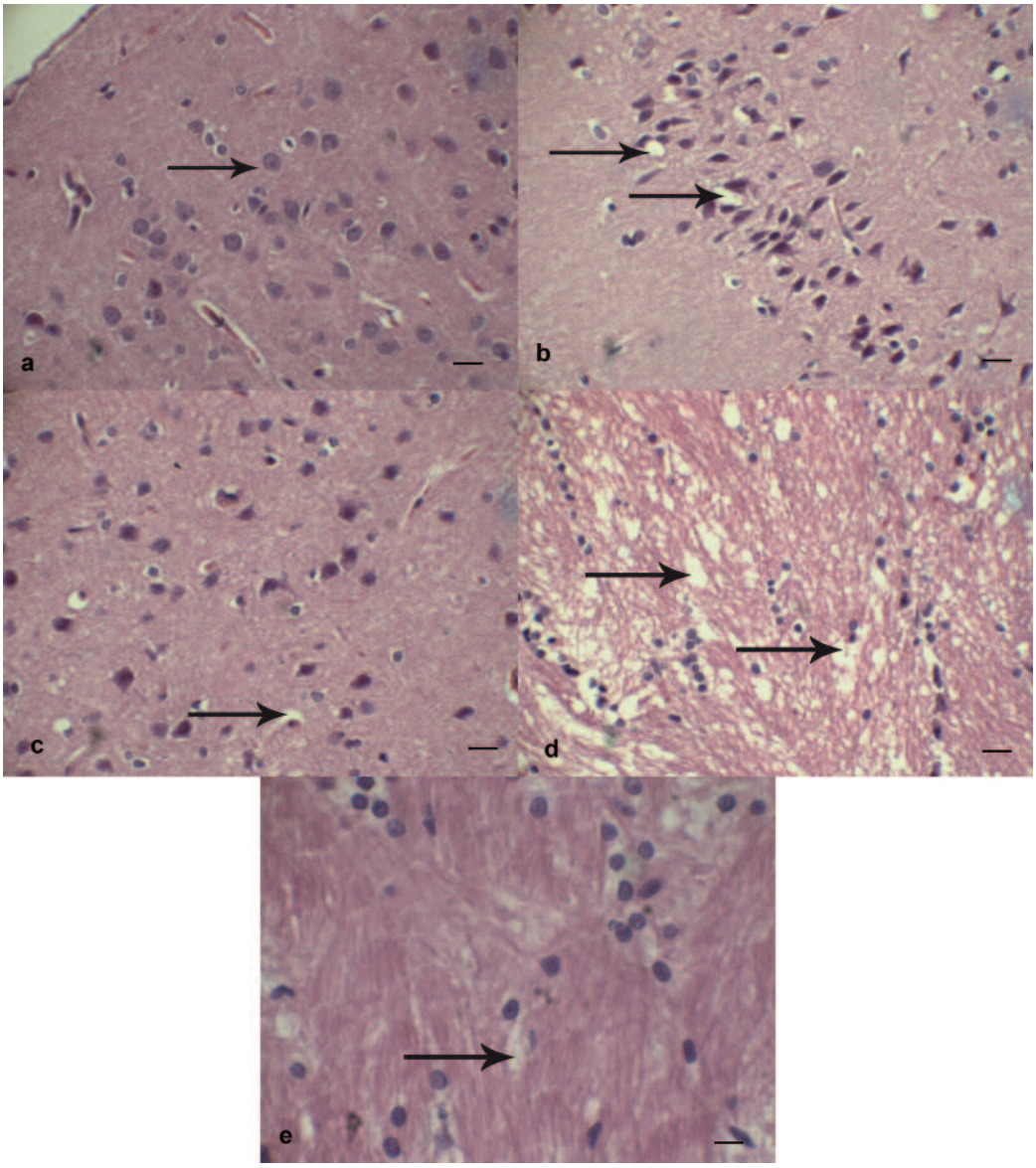

Figure 1. Neuronal morphology indicated by hematoxylin-eosin staining. ***A,*** Histological appearances of normal brain parenchyma in Sham group. Neurons have a large, round nucleus with a single prominent nucleolus (arrow). H&E, Scale Bar 20 μm. ***B,*** Edema and pyknosis in Trauma+Saline group. Scattered clusters of angular ischemic neurons with pyknotic nuclei and vacuolization (edema) in the neuropil (arrow). H&E, Scale Bar 20 μm. ***C,*** Decrease in edema (arrow) and pyknosis in Trauma+Etanercept group. H&E, Scale Bar 20 μm. ***D,*** Dense axonal swelling (arrow) in Trauma+Saline group. H&E, Scale Bar 20 μm. ***E,*** Moderate axonal swelling (arrow) in Trauma+Etanercept group. H&E, Scale Bar 20 μm.

provides further support to anti-inflammatory effect of Etanercept (Figure 1 a-c) (Table III).

When neurodegenerative changes are examined, it was found that nuclear pyknosis, nuclear hyperchromacia and cytoplasmic eosinophilia significantly increased. However, in comparison with Trauma+Saline group, no therapeutic finding was found in experiment groups. Significant improvement was observed only for axonal swelling in Trauma+Etanercept and Trauma+Saline groups ($p < 0.001$). This finding also provides further support to neuronal injury relieving effect of Etanercept (Figure 1 d, e) (Table III).

Etanercept and LiCl Decreased Neuronal and Glial Apoptosis During TBI

When TUNEL(+) motor neurons are counted in perilesional region of cortex, a significant increase in observed in number of apoptotic motor neurons in Trauma+Saline group. Although de-

Table III. Histological assessment of motor neurons in perilesional cortex and median values (25%-75% percentiles) for all groups.

		Sham	Trauma+Saline	Trauma+Etanercept	Trauma+LiCl	Trauma+Etanercept+LiCl	H and p
Histopathologic Changes	Vascular congestion	0.00 (0.00-0.00)	3.00 (3.00-3.00)	2.00 (2.00-2.00)	3.00 (3.00-3.00)	3.00 (3.00-3.00)	34.734 $p < 0.001^{A,C,D}$
	Intraparenchymal hemorrhage	0.00 (0.00-0.00)	1.00 (0.50-2.00)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	17.636 $p < 0.001^{A,D}$
	Inflammation	0.00 (0.00-0.00)	1.00 (1.00-1.50)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.00 (1.00-1.00)	34.074 $p < 0.001^{A,B,C,D}$
	Edema	0.00 (0.00-0.00)	3.00 (2.50-3.00)	2.00 (2.00-2.50)	1.00 (1.00-1.50)	1.00 (1.00-1.00)	32.497 $p < 0.001^{A,B,E}$
Neuronal Degenerative Changes	Reactive astrogliosis	0.00 (0.00-0.00)	2.00 (1.50-3.00)	2.50 (1.50-3.00)	2.00 (1.50-3.00)	2.50 (2.00-3.00)	21.018 $p < 0.001^{A,B,C,D}$
	Nuclear picnosis	0.00 (0.00-0.00)	2.50 (2.00-3.00)	1.50 (1.00-2.00)	2.00 (1.00-2.00)	1.00 (1.00-2.00)	26.470 $p < 0.001^{A,B,C}$
	Nuclear hyperchromasia	0.00 (0.00-0.00)	2.00 (2.00-3.00)	2.00 (2.00-2.50)	2.00 (2.00-2.00)	2.00 (2.00-2.00)	27.857 $p < 0.001^{A,B,C,D}$
	Cytoplasmic eosinophilia	0.00 (0.00-0.00)	2.00 (2.00-3.00)	2.00 (2.00-2.50)	2.00 (2.00-2.00)	2.00 (2.00-2.00)	27.857 $p < 0.001^{A,B,C,D}$
	Axonal swelling	0.00 (0.00-0.00)	3.00 (2.50-3.00)	2.00 (2.00-2.00)	1.00 (1.00-1.50)	1.00 (1.00-1.00)	32.652 $p < 0.001^{A,B,E}$

no injury: 0, mild:1, moderate:2, dense/common:3

The statistical difference resulting from the comparison of the Trauma+Saline vs. Sham group (A), Trauma+Etanercept vs. Sham group (B), Trauma+LiCl vs. Sham group (C), Trauma+Etanercept+LiCl vs. Sham group (D) and Trauma+Saline vs. Trauma+Etanercept group (E).

crease was observed in number of TUNEL(+) motor neurons in Trauma+Etanercept group, it could not reach statistically significance level in comparison with Trauma+Saline group. In LiCl alone or combination of LiCl and Etanercept in post-traumatic period, a slight decrease is observed in number of apoptotic motor neurons in comparison with Trauma+Saline group. Findings were not statistically significant although we observed anti-apoptosis effect of LiCl (Figure 2 a-e) (Table IV).

Etanercept and LiCl Decreased GFAP Expression During TBI

When the GFAP(+) protoplasmic astrocytes were counted in the perilesional region of the cortex, GFAP(+) cell number was seriously high in Trauma+Saline group and statistically significant increase was observed in comparison with Sham group ($p < 0.001$). In comparison with Trauma+Saline group, a statistically significant decrease was present in GFAP(+) cell count of Trauma+Etanercept, Trauma+LiCl and Trauma+Etanercept+LiCl groups ($p < 0.001$) (Figure 3 a-e) (Table IV).

Etanercept ve LiCl Decreased TNF Activity During TBI

When TNF-α immunoreactivity was evaluated, a statistically significant decrease was noted in TNF-α immunoreactivity of motor neurons in Travma+Etanercept group in comparison with Trauma+Saline group ($p < 0.001$) (Figure 4 a-d). When Trauma+Etanercept group was compared with combination group, the group of Etanercept alone had significant decrease in TNF-α immunoreactivity ($p < 0.001$) (Figure 4 e, f). Such effect of Etanercept, a TNF-α antagonist, is an expected finding.

When glial immunoreactivity was examine, glial immunoreactivity significantly decreased in Trama+Etanercept group in comparison with Trauma+Saline group ($p < 0.001$). LiCl did not have any suppressive effect on TNF-α immunoreactivity. In comparison with Trauma+Saline group, statistically significant decrease was observed again in Trauma+Etanercept+LiCl group ($p < 0.001$) (Figure 5 a-c).

When endothelial cells were taken into consideration, there was decrease in TNF-α immunoreactivity in Trauma+Etanercept group in comparison with Trauma+Saline group. When comparison was made between Trauma+LiCl group and Trauma+Etanercept+LiCl group, a significant

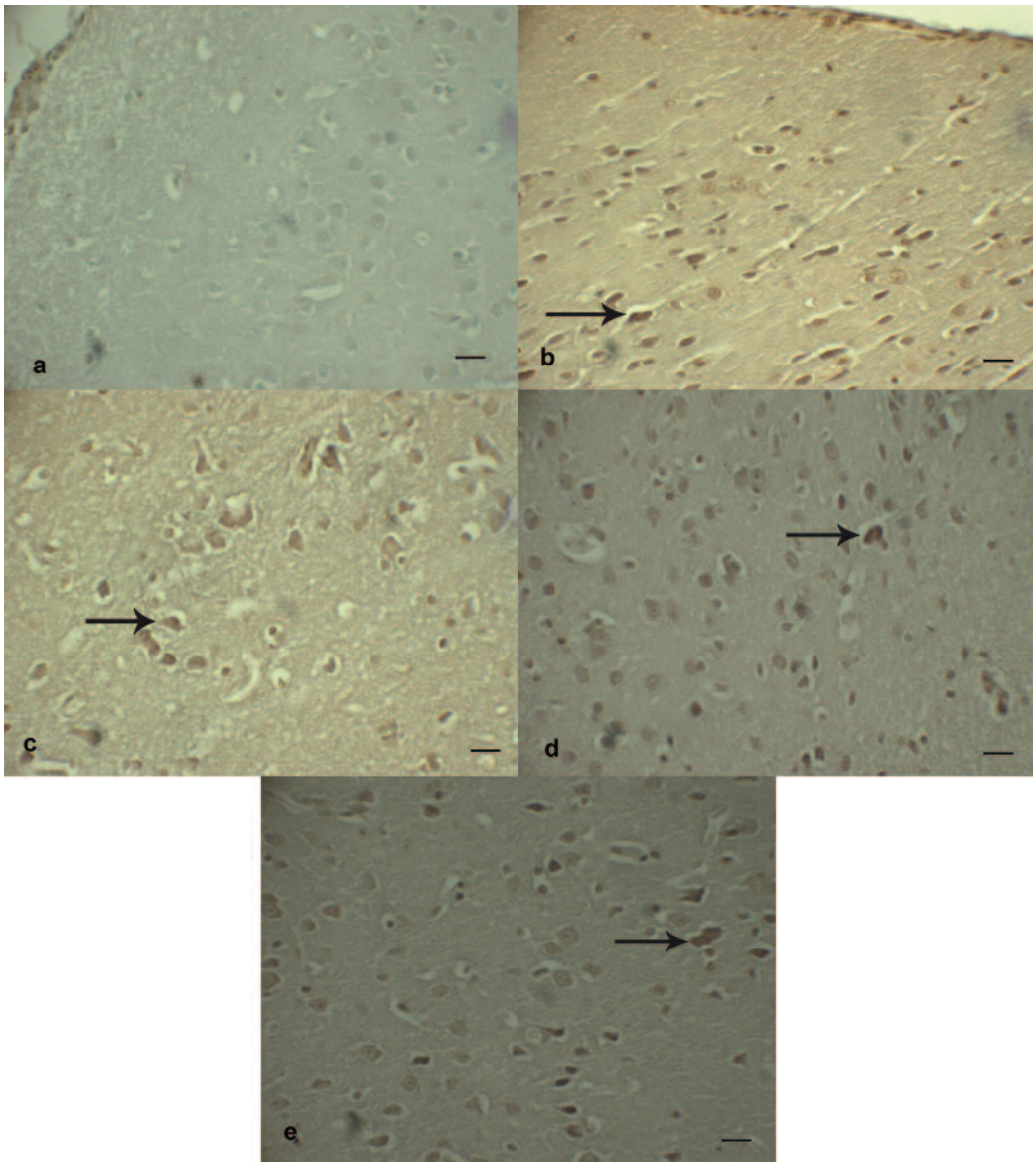

Figure 2. TUNEL immunohistochemistry staining in the perilesional region of the cortex of the brain following TBI. ***A,*** Sham group rats showing few/not TUNEL(+) apoptotic cells. TUNEL, Scale Bar 20 μm. ***B,*** Trauma+Saline group rats showing more TUNEL(+) apoptotic cells (arrow) than Sham and other groups. TUNEL, Scale Bar 20 μm. ***C,*** TUNEL(+) apoptotic cells (arrow), in Trauma+Etanercept group. TUNEL, Scale Bar 20 μm. ***D,*** TUNEL(+) apoptotic cells (arrow), in Trauma+LiCl group. TUNEL, Scale Bar 20 μm. ***E,*** TUNEL(+) apoptotic cells (arrow), in Trauma+Etanercept+LiCl group. TUNEL, Scale Bar 20 μm.

decrease was observed in TNF-α immunoreactivity in combined group ($p < 0.001$). We believe that decrease was secondary to effect of Etanercept. When comparison was made between Trauma+Saline group and Trauma+Etanercept+LiCl group, statistically significant decrease was observed again in TNF-α immunoreactivity in combined group ($p < 0.001$) (Figure 5 d, e). When comparison was made between Trauma+Etanercept group and Trauma+LiCl group, statistically significant decrease was observed again in TNF-α immunoreactivity in Trauma+LiCl group ($p < 0.001$).

In general terms, when results are examined, we found that TNF-α immunoreactivity increase in perilesional cortex 24 hours after TBI. TNF-α activity decreased after Etanercept and LiCl were administered, and tissue edema and axonal swelling were also decreased, resulting with facilitation of alleviating brain damage.

Discussion

TBI is a leading cause of death and disability worldwide[14]. Immediately following TBI, the direct trauma and lack of blood flow cause necrotic neuronal death[4,10,23]. Neuronal cell death, including apoptosis, in general peaks after 24 to 48 h after transient middle cerebral artery occlusion[44]. Apoptosis in the traumatically injured brain occurs not only at the impact site but also as a result of secondary brain insults such as intracranial hypertension, hypoxia, or disturbances of microcirculation[3]. Genovese et al[45] using the TUNEL coloration they have clearly confirmed

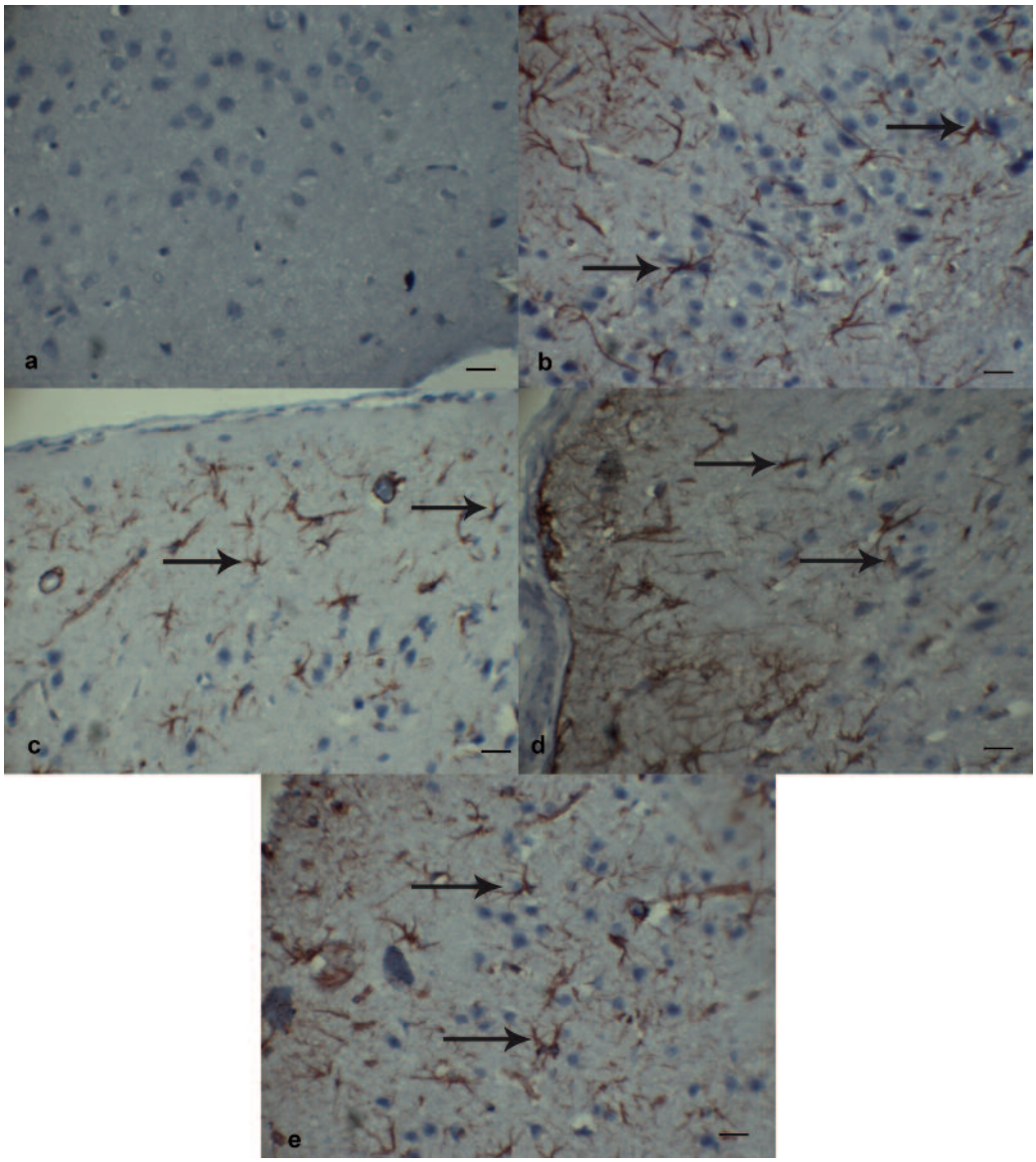

Figure 3. Immunohistochemistry for GFAP in the perilesional region of the cortex of the brain following TBI. ***A,*** Appearance of protoplasmic astrocytes in Sham group. Note that the territories of astrocyte processes do not overlap and that many astrocytes do not express detectable levels of GFAP. GFAP, Scale Bar 20 μm. ***B,*** Severe diffuse reactive astrogliosis with pronounced up regulation of GFAP expression, astrocyte hypertrophy, astrocyte proliferation and pronounced overlap of astrocyte processes resulting in disruption of individual astrocyte domains in Trauma+Saline group (arrow). GFAP, Scale Bar 20 μm. ***C,*** Mildly reactive astrogliosis in Trauma+Etanercept group. Note that the territories of protoplasmic astrocyte processes do not overlap (arrow) and that many astrocytes do not express detectable levels of GFAP. GFAP, Scale Bar 20 μm. ***D,*** Moderately reactive astrogliosis in which most (if not all) protoplasmic astrocytes have up regulated expression of GFAP and exhibit cellular hypertrophy, but with preservation of individual astrocyte domains and without pronounced overlap of astrocyte processes in Trauma+LiCl group (arrow). GFAP, Scale Bar 20 μm. ***E,*** GFAP(+) protoplasmic astrocytes in Trauma+Etanercept+LiCl group. GFAP, Scale Bar 20 μm.

Table IV. Median values of total TUNEL(+) motor neurons in perilesional cortex and total GFAP(+) protoplasmic astrocytes for all groups (25%-75% percentiles).

Groups	Tunel(+)	GFAP(+)
Sham	90.00 (81.00-134.00)	276.00 (248.50-276.00)
Trauma+Saline	1286.50 (479.00-2289.00)	2021.00 (1569.00-2632.00)
Trauma+Etanercept	1107.50 (879.00-1369.50)	930.00 (770.50-1017.00)
Trauma+LiCl	795.50 (663.00-968.50)	996.50 (891.00-1182.00)
Trauma+Etanercept+LiCl	781.00 (541.50-961.50)	1060.00 (693.00-1170.50)
H and *p*	21.035 $p < 0.001^{A,B,C,D}$	18.941 $p < 0.001^{A,B,C,D,E,F,G}$

The statistical difference resulting from the comparison of the Trauma+Saline vs. Sham group (A), Trauma+Etanercept vs. Sham group (B), Trauma+LiCl vs. Sham group (C), Trauma+Etanercept+LiCl vs. Sham group (D), Trauma+Saline vs. Trauma+Etanercept group (E), Trauma+Saline vs. Trauma+LiCl group (F), and Trauma+Saline vs. Trauma+Etanercept+LiCl group (G).

that apoptosis is present in the perilesional area after spinal cord injury (SCI) and that the combination treatment with etanercept and dexamethasone (DEX) attenuates the degree of apoptosis. Moreover, this attenuation is due to the prevention of loss of the anti-apoptotic way and reduction of pro-apoptotic pathway activation with a mechanism still to discover. In the current study, we determined apoptosis in perilesional cortical region of brain tissue with TUNEL staining method following TBI. Our findings were not statistically significant in comparison with Trauma+Saline group, although severity of apoptosis decreased in Etanercept, LiCl and Etanercept+LiCl groups.

Neutrophils, macrophages and activated microglia may act as scavenger cells to remove cellular debris and release cytotoxic or neurotrophic molecules into the injured tissue[7-9]. However, even greater apoptotic neuronal loss occurs hours and days later, caused by secondary injury from cerebral ischemia/hypoxia as well as inflammatory and oxidative stress. Inhibition of cytokine production by anti-inflammatory agents, including minocycline and statins, has been shown to reduce TBI. Therefore, the major focus of TBI research should be protection of neurons from apoptotic cell death by reducing the secondary injury of inflammation and oxidative stress[12].

Brain inflammation represents only one of the numerous processes activated after TBI. The major effectors in this cascade are the pro-inflammatory cytokines that are usually released within minutes after challenge because they are stored intracellular as precursor proteins eventually modified into active molecules[3]. Our study showed that cortical levels of TNF-α was significantly induced by TBI at 24 h after trauma. We have clearly demonstrated, by immunohistochemistry, a significant increase of positive staining for TNF-α in Trauma+Saline group compared with Sham group animals. On the contrary, no significant expression of TNF-α was observed in the brain tissue sections obtained from Etanercept-treated rats. In this study, we observe that Etanercept treatment reduces the expression of TNF-α. Finally, in this work, we demonstrate that Etanercept treatment significantly reduced the TBI-induced brain tissues alteration and improve edema and axonal swelling.

Histopathological studies following severe closed head injury in our model demonstrated a diffuse injury that affected the perilesional cortex. Vascular congestion was noted throughout the brain. The presence and pattern of diffuse vascular congestion, intraparenchymal hemorrhage, inflammation, edema, and reactive astrogliosis were similar at the high levels of injury. In our diffuse injury model, rats in the Trauma+Saline group had appreciable cerebral edema noted histologically 24 hours after injury.

Axonal injury is one of the most common pathologies resulting from brain trauma, where the extent of axonal damage is thought to play a major role in outcome. Initial swelling and further damage of injured axons may result from a change in ionic homeostasis. As a result of these ionic changes, osmotic swelling of axons may occur shortly following injury due to sodium influx, while the increase in intracellular calcium may induce the deleterious activation of proteases leading to additional cytoskeletal damage. Although these processes are thought to play important roles in the ultimate demise of injured axons, swelling alone is not considered the principle feature of traumatically injured axons. Rather, accumulation of axonal transport proteins in swollen regions of axons is the clear signature of catastrophic damage[13]. Beginning from only hours following trauma, both disconnected axonal bulbs and still-con-

Table V. Median values of TNF-α(+) immunoreactivity in motor neurons in perilesional cortex, glia and endothelium for all groups (25%-75% percentiles).

		Sham	Trauma+Saline	Trauma+Etanercept	Trauma+LiCl	Trauma+Etanercept+LiCl	H and *p*
Neuron	0	47.20 (41.60-52.95)	18.20 (14.85-46.80)	57.40 (53.25-65.75)	27.40 (23.90-9.40)	45.80 (42.85-54.70)	7.678 $p = 0.104$
	1	2.00 (1.30-2.65)	3.40 (0.00-6.25)	1.40 (0.85-3.05)	4.80 (4.65-12.15)	18.20 (14.85-46.80)	15.243 $p = 0.004^{D,F}$
	2	0.00 (0.00-0.45)	26.00 (18.45-26.65)	0.40 (0.00-5.90)	6.00 (5.80-12.05)	3.80 (1.20-8.15)	16.179 $p = 0.003^{A,E}$
Glia	0	20.60 (19.05-29.45)	8.80 (5.65-9.75)	14.40 (12.20-17.40)	15.00 (11.10-17.00)	16.40 (13.75-16.95)	9.027 $p < 0.001^{A,B,C,D}$
	1	4.20 (3.30-5.05)	0.00 (0.00-0.00)	3.60 (2.60-4.55)	0.00 (0.00-0.050)	0.00 (0.00-0.40)	19.870 $p < 0.001^{A}$
	2	1.80 (0.00-6.05)	13.20 (11.60-15.40)	5.40 (2.50-9.10)	10.00 (8.05-15.90)	6.40 (5.80-6.95)	8.387 $p < 0.001^{A,C,E,H}$
Endothelium	0	3.80 (2.70-6.15)	1.00 (0.00-2.00)	2.20 (1.95-4.25)	0.40 (0.15-0.70)	1.60 (0.70-2.60)	14.009 $p = 0.007^{C}$
	1	0.00 (0.00-2.15)	0.00 (0.00-0.00)	7.40 (5.75-11.55)	1.80 (0.00-1.85)	8.20 (6.35-9.35)	17.710p < $0.001^{B,D,E,G,H,J}$
	2	0.00 (0.00-0.45)	3.20 (1.20-6.60)	1.40 (0.00-2.50)	6.60 (6.20-7.00)	0.00 (0.00-1.80)	14.208 $p = 0.007^{C,G}$

0: no immunoreactivity; 1: trace immunoreactivity; 2: increased immunoreactivity

The statistical difference resulting from the comparison of the Trauma+Saline vs. Sham group (A), Trauma+ Etanercept vs. Sham group (B), Trauma+LiCl vs. Sham group (C), Trauma+Etanercept+LiCl vs. Sham group (D), Trauma+Etanercept vs. Trauma+Saline group (E), Trauma+Etanercept vs. Trauma+Etanercept+LiCl group (F), Trauma+LiCl vs. Trauma+Etanercept+LiCl group (G), Trauma+Etanercept+LiCl vs. Trauma+Saline group (H) and Trauma+Etanercept vs. Trauma+LiCl group.

nected axonal swellings can be identified[13,14] In the current study, we observed axonal swelling in Trauma+Saline group. The finding resulted from change in ionic homeostasis following TBI. In Trauma+Etanercept group, we observed that axonal swelling significantly decreased.

Microtubule-associated protein Tau is localized primarily in the axonal compartment of neurons. Functionally, Tau binds to axonal microtubules resulting in the formation of microtubule bundles. These bundles form important structural elements of the axonal cytoskeleton and are critical elements in the axoplasmic flow of proteins between the nerve terminal and neuronal cell body[15]. Tau protein that can be measured in serum is released from CNS neurons after TBI. Liliang et al[5] show that Tau protein levels also rise slightly in Sham group rats in the first 6 h after TBI. However, the rise in Sham group did not reach statistical significance. As with any surgery, there was possible some additional injury to brain and Tau protein levels increased. This increase in total Tau is consistent with studies employing other biomarkers such as c-Tau, S-100 and neuron-specific enolase, where increased levels were observed within 6 h after TBI in rats[5]. Wunderlich et al[16] reported that Tau protein concentrations in serum showed a continuous increase from admission to last measurement 120 h after stroke and were elevated in 27% of their patients within the first day and in 41% until day 5. Tau protein levels rapidly elevate in the CSF after TBI. The peak has been reported after 2 days or 1 week. Although serum Tau protein can be measured following TBI, the peak and progression are unclear[5,15,16]. In the current study, statistically significant increase in serum Tau level 24 hours after TBI reflects both extensive neuronal damage in the brain as well as a compromised BBB. However, we determined that post-TBI Etanercept and LiCl administration did not significantly decrease serum Tau level.

Hypertrophic astrocytes with prominent immunoreactive processes were scattered diffusely throughout the region of cell body injury. These changes, which appeared to involve all astrocytes in the area, are consistent with classic astrocytic hypertrophy in response to local injury[46]. At the immediate vicinity of injury, reactive astrocytes interweave their processes to form a barrier termed “anisomorphic gliosis”. This glial scar can be an impediment to regenerating axons[4,7-12]. Although the presence of glial activation in the injured brain certainly represents a disturbance of

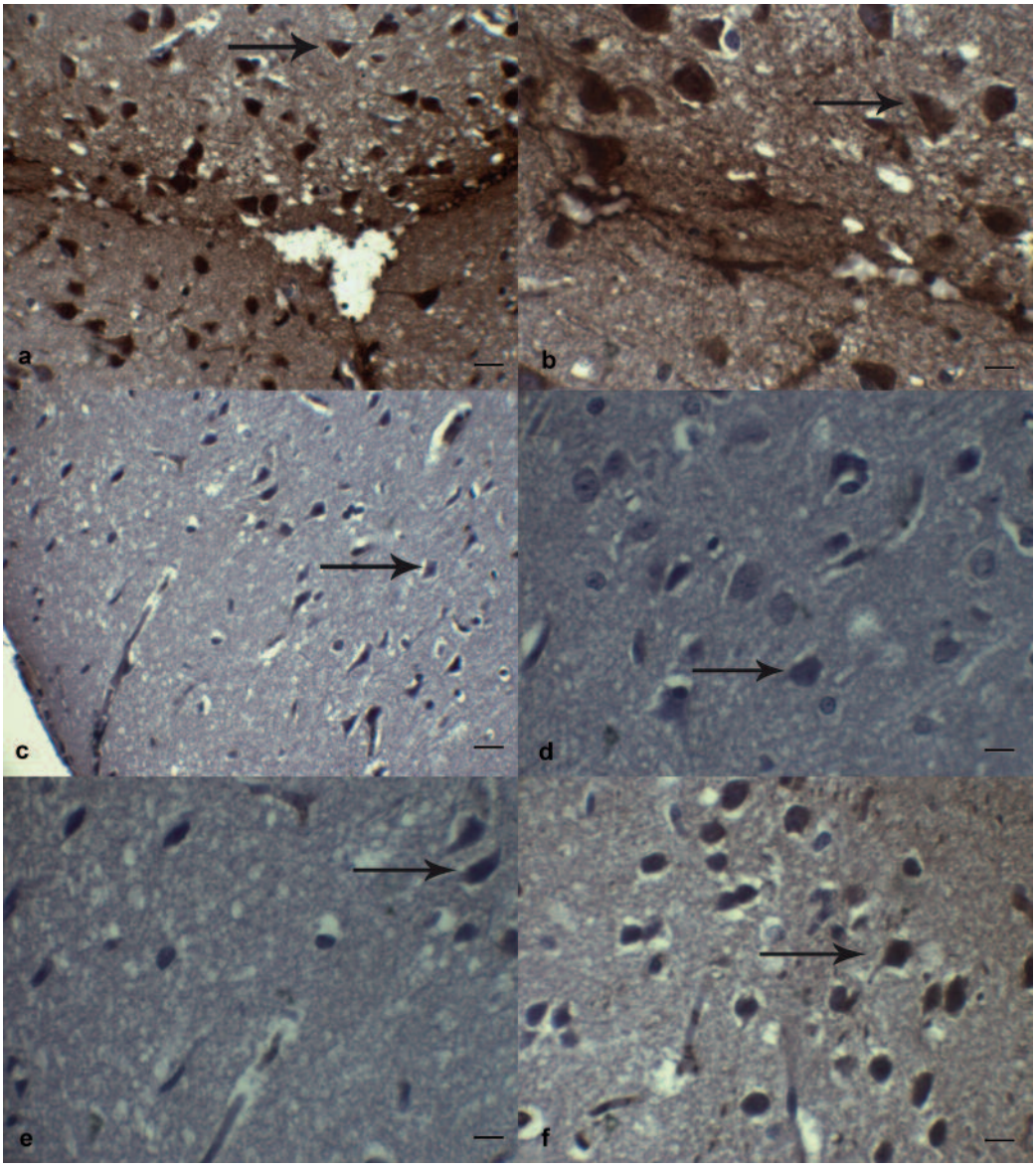

Figure 4. Photomicrographs showing TNF-α immunoreactivity at motor neurons in the perilesionel cortex. ***A,*** Note that there was a marked rise in the number of TNF-α positive cells at motor neurons (arrow) in Trauma+Saline group. TNF-α, Scale Bar 20 μm. ***B,*** A substantial increase in TNF-α production was found at motor neurons (arrow) in Trauma+Saline group. TNF-α, Scale Bar 10 μm. ***C,*** Treatment with Etanercept significantly reduced in TNF-α immunoreactivity at motor neurons (arrow) in Trauma+Etanercept group. TNF-α, Scale Bar 20 μm. ***D,*** The number of TNF-α positive cells in motor neurons (arrow) was significantly reduced in Trauma+Etanercept group. TNF-α, Scale Bar 10 μm. ***E,*** The number of TNF-α positive cells at motor neurons (arrow) was significantly reduced in Trauma+Etanercept group compared with Trauma+Etanercept+LiCl group TNF-α, Scale Bar 10 μm. ***F,*** Significantly reduced TNF-α immunoreactivity at motor neurons (arrow) in Trauma+Etanercept+LiCl group. TNF-α, Scale Bar 10 μm.

normal brain physiology or predictor of a pathological condition, it is still controversial as to whether reactive gliosis is harmful or beneficial to the acutely injured brain[18]. Reactive astrocytes perform roles essential for the preservation of neural tissue and restriction of inflammation after mild or moderate focal brain injury, and thus play a major part in determining outcome after TBI. The roles played by reactive astrocytes after TBI are not well understood. Because reactive astrocytes are ubiquitous in damaged CNS tissue, they are often regarded as uniformly harmful, causing toxic edema, provoking inflammation, releasing cytotoxins and forming scars that serve no pur-

pose but to inhibit axonal regeneration. In the context of moderate contusive brain injury reactive astrocytes were not harmful, but rather played roles essential for preserving cortical neurons and tissue integrity, and for restricting post-injury inflammation. Reactive astrocytes play important roles in restricting the infiltration of inflammatory cells into viable neural tissue after CNS injuries in vivo. In this regard it is of interest that astrocytes may be lost early after TBI[2]. TBI is accompanied by secondary or delayed brain edema and these edema processes are also believed to participate in the pathogenesis of TBI[47]. Our findings demonstrated that reactive astrocytes played major role in post-TBI period. Post-TBI enhanced reactive astrogliosis antagonized edema and inflammation in all groups and it had protective effect on cortical neurons.

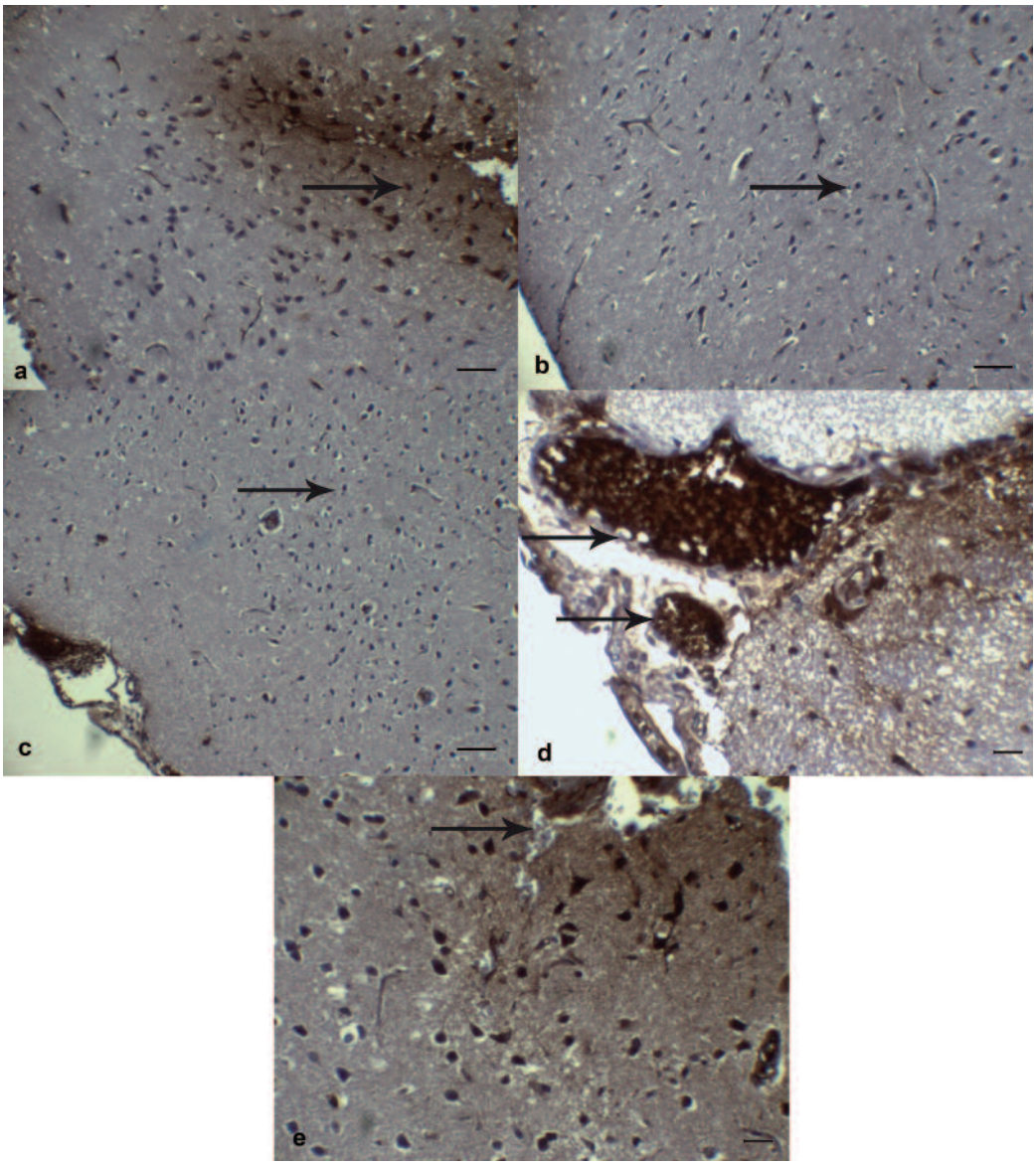

Figure 4. TNF-α immunoreactivities at glial and endothelial cells in the perilesionel cortex. ***A,*** A substantial increased TNF-α expression was found at glial cells (arrow) in Trauma+Saline group.TNF-α, Scale Bar 50 μm. ***B,*** Treatment with Etanercept significantly reduced in TNF-α immunoreactivity at glial cells (arrow) in Trauma+Etanercept group.TNF-α, Scale Bar 50 μm. ***C,***The combination therapy with Etanercept and LiCl significantly reduced the TNF-α immunreactivity at glial cells (arrow). TNF-α, Scale Bar 50 μm. ***D,*** Significant increased TNF-α expression was found at endothelial cells (arrow) in Trauma+Saline group.TNF-α, Scale Bar 20 μm. ***E,*** TNF-α immunoreactivity significantly reduced at endothelial cells (arrow) in Trauma+Etanercept+LiCl group.TNF-α, Scale Bar 20 μm.

The astrocytic reaction described above may play a role in the protection of the cortex after injury. It is known that astrocytes can synthesize trophic factors. These neurotrophins in turn may protect the surrounding neurons. Thus, it is likely that the phenomenon depicted here could be part of a series of cellular events that may result in neuronal protection[48]. Under normal condition, the BBB gates the entrance of immune cells into the CNS. Barrier disruption is associated with uncontrolled inflammatory cell entry into the CNS parenchyma. Astrocytes are considered to play a prominent role in controlling this process, since their end-feet processes are in intimate contact with the brain micro-vessels and are known to form an essential functional component of the BBB[22]. In the current study, we observed that glial cells, which are located around micro-vessels, intensely expressed TNF-α 24 hours after TBI and number of glial cells rapidly increased. We believed that this finding may play a significant role in impairment of BBB and inducing cerebral inflammatory response after TBI.

GFAP is an astrocyte specific intermediate filament protein whose expression is required for fibrous astrocyte normal function including maintenance of CNS white matter and BBB integrity. Increased GFAP expression is a hallmark of reactive astrocytes and this cytoskeletal protein contributes to a barrier effect of the glial scar for axonal extension[2,6,17,18]. Though not all astrocytes express GFAP, those reacting to injury show a rise in the content of these filaments. This makes GFAP immunohistochemistry a very useful marker to study gliosis. Different models of brain injury have been studied using this marker[48]. These data correlate with an elevation in GFAP gene expression (mRNA) which could be detected in response to mild cortical contusions in animals[19,20]. In this report, serum GFAP level significantly increased in Trauma+Saline group. As indicated by tissue immunohistochemistry, GFAP(+) finding of Trauma+Saline group significantly increased in Etanercept, LiCl and Etanercept+LiCl groups in comparison with Trauma+Saline group.

The brain has been traditionally described as an "immune-privileged" organ due to its isolation from the peripheral immune system by the BBB. However, recent brain injury studies[18] have indicated that resident brain cells are capable of synthesizing a wide variety of pro-inflammatory mediators necessary for mounting a neuroinflammatory response. The inflammatory response in the CNS involves the participation of different cellular types of the immune system and resident cells of the CNS, adhesion molecules, cytokines, and chemokines among other proteic components. During neuroinflammation, chemotaxis is an important event in the recruitment of cells to the CNS. If this process is not controlled but is prolonged, inflammation loses its repairing function and can be the cause of damage[28].

In addition to chemokines, various cytokines have also been reported to be expressed following TBI, including TNF-α associated with activated microglia and astrocytes that may initiate the inflammatory process and are thought to be the primary sources of cytokines and chemokines that play a role in brain inflammatory responses[4,22]. TNF-α is known to be bioactive both as a soluble and as a transmembrane protein. TNF-α is released during and involved in the inflammatory response following TBI. Elevated levels of TNF-α have been associated with the pathological effects of a variety of infectious, neurological, neurodegenerative, and neurotoxic conditions. In contrast to these findings, however, some evidence suggests that TNF-α also plays a neuroprotective role following TBI. TNF-α has also been reported to participate actively in BBB breakdown as well as acute post-traumatic apoptotic and necrotic cell death[1,6,17,22,23,47,49].

Both necrotic and apoptotic mechanisms of cell death after SCI have been well and extensively characterized in animal SCI models. It is has been thought that microglial cells might be the source of cytotoxic cytokines, such as TNF-α, that kill oligodendrocytes. Within 1 h after SCI, increased synthesis and/or secretion of TNF-α is detectable at the injury site[28]. Pettigrew et al[50] emphasized TNF-α is frequently undetectable in quiescent neural cell populations, becoming up-regulated in neurons and astroglial cells only after ischemic or post-traumatic stress. They speculate that the greater infarct volume observed in the transgenic animal resulted from chronic overexpression of TNF-α protein within the brain, causing receptor-mediated caspase activation that promoted neuronal apoptosis. Mao et al[51] found that TNF-α expression is increased in neonatal stroke and may be involved in neuronal apoptosis. TNF-α expression is increased in a wide range of CNS disorders such as trauma and ischemia. In this study, TNF-α expression increased in the ischemic cortex after stroke. In the current study, paralleled increase in remarkable TNF-α(+) and TUNEL(+) staining in Trau-

ma+Saline group indicated that TNF-α was involved in apoptotic mechanisms. After Etanercept, a TNF-α antagonist, is administered, TNF-α(+) staining and decreased apoptosis had intercorrelating results.

In particular, both IL-1β and TNF-α mRNA levels have been shown to peak 1-3 h following experimental TBI in rats[18]. In animal models of cerebral ischemia, high levels of TNF-α have been found after global and focal ischemic injury[50]. Hang et al[52] showed that TNF-α and IL-6 levels were significantly increased in the surrounding area of injured brain by 3 h, maximal at 24 h and lasted for 7 days following TBI. Chen et al. demonstrated that pre-administration of lovastatin significantly reduced post-CCI TNF-α and IL-1β mRNA and protein levels. The decrease in cytokine levels was paralleled by a reduction of brain damage and neurological deficits. TNF-α directly disturbs BBB integrity, leading to cerebral edema and leukocyte infiltration whereas inhibiting TNF-α synthesis or neutralizing TNF-α activity reduces development of edema and neuronal degeneration and improves neurological outcome in various brain injury models. Our results are consistent with that of above specified study. In the current study, after Etanercept and LiCl were administered, decreased TNF-α activity was associated with tissue edema and axonal swelling and it facilitated regression of cerebral damage.

Kita et al[47] indicated that TNF-α was detected 1 h after impact at the glial cells such as astrocytes, and inflammatory cells such as intravascular leukocyte and perivascular monocyte-derived cells in the hippocampus and parasagittal cortex. In addition immunoreactions for TNF-α were detected at the apical plasma membrane of the capillary endothelium and lysosomes of the edematous changes occurred in the vascular feet of astrocytes 1 h after impact. In the current study, TNF-α activity of glial and endothelial cells is consistent with results of this study. Histopathologically remarkable vascular congestion, intraparenchymal hemorrhage, inflammation, neuronal necrosis, reactive astrogliosis and edema were observed 24 hours after TBI. In addition, neuronal cell bodies were shrunken and eosinophilic, and nuclei were pyknotic. Astrocytes increase in number and size in response to injury and swell after brain injuries after TBI. GFAP of immunohistochemical analysis demonstrated astrocyte enlargement and increased number in the perilesional cortex. TBI activates an inflammatory reaction initiated by the release of TNF-α has been observed in the rat brain. The present study also demonstrates that TNF-α contribute to brain inflammation following the mechanical traumatic damage in the rat brain. Etanercept achieved a significant decrease in edema and axonal swelling. LiCl alone and combination of LiCl and Etanercept maintained the decrease.

Clinical investigations have supported that elevated TNF-α levels in human serum and CSF after TBI. These studies have also provided evidence that the pharmacological inhibition of TNF-α after head injury mediates neuroprotective effects. Studies in animals have provided key evidence that antagonizing TNF-α is a viable therapeutic strategy. Biological agents that are currently available include three agents that decrease the activity of TNF-α (infliximab, adalimumab, and etanercept). The major therapeutic goal when administering TNF-α antagonists is to eliminate the surplus of TNF-α from the circulation and from sites of inflammation[28,45]. There are few reports on the head truma with Etanercept[1,12,53]. These studies are promising, but some author criticized that this drug has a limited transportation to the neural tissue due to the BBB[54]. However, the BBB disruption appear after the head trauma but it can be used differently for poorly transporting drugs[55-57]. Marchand et al[58] show that immediate treatment with Etanercept reduces microglial activation. Genovese et al[45] have demonstrated that treatment with Etanercept at a dose of 5 mg/kg reduced the post-inflammatory reaction as well as the motor dysfunction associated with SCI. They have clearly demonstrated a significant increase of TNF-α and IL-1β in SCI. On the contrary, no significant expression of TNF-α and IL-1β was observed in the spinal cord sections obtained from SCI-operated mice which received the combination treatment with Etanercept and DEX. In the current study, TNF-α expression intensely increased in neuron, glial and endothelium in Trauma+Saline group 24 hours after TBI. In addition, similar to results of other investigators, we observed that TNF-α positivity significantly decreased in endothelial cells after Etanercept, TNF-α antagonist, was administered. Significant decrease in TNF-α positivity was also observed in neurons and glial cells. We believe that TNF-α accompanies pathogenesis of secondary brain damage, which involves astrogliosis and apoptosis. These results suggest that TNF-α might play a central role in the inflammatory response that leads to secondary insults after TBI.

Lithium have neuroprotective and neuroregenerative properties[59]. Lithium has emerged as a robust neuroprotective agent that prevents apoptosis of neurons through multiple mechanisms. Lithium up-regulates anti-apoptotic Bcl-2 and down-regulates pro-apoptotic p53 and Bax. Lithium has an astonishing range of activity on the cells. In fact, it appears almost designed to turn on multiple cellular mechanisms and genes that protect the cells against toxins, stress, ischemia, and injury. At the same time that it protects cells, it also turns genes on for growth factors, cellular repair, and regeneration[29]. Su et al[30] showed that lithium treatment at the therapeutic concentration (1 mM) is able to enhance the neuronal differentiation of NPCs *in vitro* and after transplantation into the avulsed ventral horn of adult rats. They revealed the enhancing effect of Lithium on proliferation and neuronal differentiation of NPCs in the adult rat spinal cord. In this paper, we observed a sigificant decrease in TNF-α positivity and GFAP positivity after LiCl was administered. Those results suggest that LiCl acts like neuroprotective and neuro-regenetative agents and it accompanies decreased apoptosis in LiCl group.

Conclusions

This study is planned to prevent post-traumatic secondary tissue damages in rat diffuse severe brain injury model, which is induced by alone or combined administration of Etanercept and LiCl. Studies in animals have provided key evidence that antagonizing TNF-α is a viable therapeutic strategy. Biological agents that are currently available include three agents that decrease the activity of TNF-α (infliximab, adalimumab, and etanercept). The major therapeutic goal when administering TNF-α antagonists is to eliminate the surplus of TNF-α from the circulation and from sites of inflammation. In this study, we demonstrate that Etanercept treatment significantly reduced the TBI-induced brain tissues alteration, reduced the expression of TNF-α and improve edema and axonal swelling. Lithium have neuroprotective and neuroregenerative properties. In this study, we observed a significant decrease in TNF-α and GFAP positivity after LiCl was administered. Those findings suggest that LiCl acts like neuroprotective and neuro-regenetative agents and it accompanies decreased apoptosis in LiCl group. Apoptosis continued decreasing when LiCl was administered alone or in combination with Etanercept. In conclusion, the combination therapy with Etanercept and LiCl decreased neuronal degeneration and alleviated secondary tissue damage in post-traumatic period.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

References

1) Chio Cc, Lin Jw, Chang Mw, Wang Cc, Kuo Jr, Yang Cz, Chang Cp. Therapeutic evaluation of etanercept in a model of traumatic brain injury. J Neurochem 2010; 115: 921-929.
2) Myer Dj, Gurkoff Gg, Lee Sm, Hovda Da, Sofroniew Mv. Essential protective roles of reactive astrocytes in traumatic brain injury. Brain 2006; 129: 2761-2772.
3) Chen G, Shi J, Hu Z, Hang C. Inhibitory effect on cerebral inflammatory response following traumatic brain injury in rats: A potential neuroprotective mechanism of N-Acetylcysteine. Mediat Inflamm 2008; Article ID 716458, 8 pages doi: 10.1155/2008/716458.
4) Das M, Mohapatra S, Mohapatra SS. New perspectives on central and peripheral immune responses to acute traumatic brain injury. J Neuroinflamm 2012; 9: 236.
5) Liliang Pc, Liang Cl, Lu K, Wang Kw, Weng Hc, Hsieh Ch, Tsai Yd, Chen Hj. Relationship between injury severity and serum tau protein levels in traumatic brain injured rats. Resuscitation 2010; 81: 1205-1208.
6) Chen Y, Swanson Ra. Astrocytes and brain injury. J Cerebral Blood Flow Met 2003; 23: 137-149.
7) Yang L, Jones Nr, Blumbergs Pc, Heuvel Cvd, Moore Ej, Manavis J, Sarvestani Gt, Ghabrie Mn. Severity-dependent expression of pro-inflammatory cytokines in traumatic spinal cord injury in the rat. J Clin Neurosci 2005; 12: 276-284.
8) Neher Md, Weckbach S, Flierl Ma, Huber-Lang Ms, Stahel Pf. Molecular mechanisms of inflammation and tissue injury after major trauma-is complement the "bad guy"? J Biomed Sci 2011; 18: 90.
9) Schmidt Oi, Infanger M, Heyde Ce, Ertel W, Stahel Pf. The role of neuroinflammation in traumatic brain injury. Eur J Trauma 2004; 30: 135-149.
10) Park E, Bell Jd, Baker Aj. Traumatic brain injury: Can the consequences be stopped? Can Med Assoc J 2008; 178: 1163-1170.
11) Yu Ch, Yhee Jy, Kim Jh, Im Ks, Kim Nh, Jung Di, Lee Hc, Chon Sk, Sur Jh. Pro- and anti-inflammatory cytokine expression and histopathological characteristics in canine brain with traumatic brain injury. J Vet Sci 2011; 12: 299-301.

12) Khan M, Im Yb, Shunmugavel A, Gilg Ag, Dhindsa Rk, Singh Ak, Singh I. Administration of S-nitrosoglutathione after traumatic brain injury protects the neurovascular unit and reduces secondary injury in a rat model of controlled cortical impact. J Neuroinflamm 2009; 6: 32.

13) Smith Dh, Meaney Df. Axonal damage in traumatic brain injury. Neuroscientist 2000; 6: 483-495.

14) Hortobagyi T, Al-Sarraj S. The significance of diffuse axonal injury: How to diagnose it and what does it tell us? ACNR 2008; 8: 16-18.

15) Irazuzta Je, Courten-Myers G, Zemlan Fp, Bekkedal Myv, Rossi J. Serum cleaved Tau protein and neurobehavioral battery of tests as markers of brain injury in experimental bacterial meningitis. Brain Res 2001; 913: 95-105.

16) Wunderlich Mt, Lins H, Skalej M, Wallesch Cw, Goertler M. Neuron-specific enolase and tau protein as neurobiochemical markers of neuronal damage are related to early clinical course and long-term outcome in acute ischemic stroke. Clin Neurol Neurosur 2006; 108: 558-563.

17) Sriram K, Matheson Jm, Benkovic Sa, Miller Db, Luster Mi, O'callaghan Jp. Deficiency of TNF receptors suppresses microglial activation and alters the susceptibility of brain regions to MPTP-induced neurotoxicity: Role of TNF- . FASEB J 2006; 20: 670-682.

18) Williams Aj, Wei Hh, Dave Jr, Tortella Fc. Acute and delayed neuroinflammatory response following experimental penetrating ballistic brain injury in the rat. J Neuroinflamm 2007; 4: 17.

19) Hausmann R, Rieb R, Fieguth A, Betz P. Immunohistochemical investigations on the course of astroglial GFAP expression following human brain injury. Int J Legal Med 2000; 113: 70-75.

20) Zurek J, Fedora M. The usefulness of S100B, NSE, GFAP, NF-H, secretagogin and Hsp70 as a predictive biomarker of outcome in children with traumatic brain injury. Acta Neurochir (Wien) 2012; 154: 93-103.

21) Liu T, Clark Rk, Mcdonnell Pc, Young Pr, White Rf, Barone Fc, Feuerstein Gz. Tumor necrosis factor-alpha expression in ischemic neurons. Stroke 1994; 25: 1481-1488.

22) Gong C, Qin Z, Betz AL, Liu XH, Yang GY. Cellular localization of tumor necrosis factor alpha following focal cerebral ischemia in mice. Brain Res 1998; 801: 1-8.

23) Chen Sf, Hung Th, Chen Cc, Lin Kh, Huang Yn, Tsai Hc, Wang Jy. Lovastatin improves histological and functional outcomes and reduces inflammation after experimental traumatic brain injury. Life Sci 2007; 81: 288-298.

24) Kato K, Liu H, Kikuchi Si, Myers Rr, Shubayev Vi. Immediate anti-tumor necrosis factor-α (etanercept) therapy enhances axonal regeneration after sciatic nerve crush. J Neurosci Res 2010; 88: 360-368.

25) Moreland Lw, Baumgartner Sw, Schiff Mh, Tindall Ea, Fleischmann Rm, Weaver Al, Ettlinger Re, Cohen S, Koopman Wj, Mohler K, Widmer Mb, Blosch Cm. Treatment of rheumatoid arthritis with a recombinant human tumor necrosis factor receptor(p75)-Fc fusion protein. N Engl J Med 1997; 337: 141-147.

26) Umeda N, Ito S, Hayashi T, Goto D, Matsumoto I, Sumida T. A patient with rheumatoid arthritis who had a normal delivery under etanercept treatment. Intern Med 2010; 49: 187-189.

27) Lee Ej, Shin Mk, Kim Ni. A clinical trial of combination therapy with etanercept and low dose cyclosporine for the treatment of refractory psoriasis. Ann Dermatol 2010; 22: 138-142.

28) Genovese T, Mazzon E, Crisafulli C, Paola Rd, Muia C, Bramanti P, Cuzzocrea S. Immunomodulatory effects of etanercept in an experimental model of spinal cord injury. J Pharmacol Exp Ther 2006; 316: 1006-1016.

29) Young W: Review of lithium effects on brain and blood. Cell Transplant 2009; 18: 951-975.

30) Su H, Zhang W, Guo J, Guo A, Yuan Q, Wu W. Lithium enhances the neuronal differentiation of neural progenitor cells in vitro and after transplantation in to the avulsed ventral horn of adult rats through the secretion of brain-derived neurotrophic factor. J Neurochem 2009; 108: 1385-1398.

31) Marmarou A, Foda Ma, Brink W, Campbell J, Kita H, Demetriadou K. A new model of diffuse brain injury in rats. Part I: Pathophysiology and biomechanics. J Neurosurg 1994; 80: 291-300.

32) Conroy Bp, Grafe Mr, Jenkins Lw, Vela Ah, Lin Cy, Dewitt Ds, Johnston We. Histopathologic consequences of hyperglycemic cerebral ischemia during hypothermic cardiopulmonary bypass in pigs. Ann Thorac Surg 2001; 71: 1325-1334.

33) Li F, Liu Kf, Silva Md, Meng X, Gerriets T, Helmer Kg, Fenstermacher Jd, Sotak Ch, Fisher M. Acute postischemic renormalization of the apparent diffusion coefficient of water is not associated with reversal of astrocytic swelling and neuronal shrinkage in rats. Am J Neuroradiol 2002; 23: 180-188.

34) Lin Hw, Basu A, Druckman C, Cicchese M, Krady Jk, Levison, Sw. Astrogliosis is delayed in type 1 interleukin-1 receptor-null mice following a penetrating brain injury. J Neuroinflamm 2006; 3: 15.

35) Shojo H, Kibayashi K. Changes in localization of synaptophysin following fluid percussion injury in the rat brain. Brain Res 2006; 1078: 198-211.

36) Kalayci M, Unal Mm, Gul S, Acikgoz S, Kandemir N, Hanci V, Edebali N, Açikgöz B. Effect of Coenzyme Q10 on ischemia and neuronal damage in an experimental traumatic brain-injury model in rats. BMC Neuroscience 2011; 12: 75.

37) Ananiadoua Og, Drossos Ge, Bibou Kn, Palatianos Gm, Johnson Eo. Acute regional neuronal injury following hypothermic circulatory arrest in a porcine model. Interact Cardiov Th 2005; 4: 597-601.

38) Isaksson J, Hillered L, Olsson Y. Cognitive and histopathological outcome after weight-drop brain injury in the rat: Influence of systemic administration of monoclonal antibodies to ICAM-1.Acta Neuropathol 2001; 102: 246-256.

39) Lafuente Jv, Mitre B, Argandon Eg. Spatio-temporal distribution of apoptosis and the modulators thereof following a cortical microinfarct in rat brain. Neurosci Res 2007; 57: 354-361.

40) Yu Hm, Yuan Tm, Gu Wz, Li Jp. Expression of glial fibrillary acidic protein in developing rat brain after intrauterine infection. Neuropathology 2004; 24: 136-143.

41) Sofroniew Mv, Vinters Hv. Astrocytes: Biology and pathology. Acta Neuropathol 2010; 119: 7-35.

42) Weibenberger Ca, Varrallyay C, Raslan F, Kleinschnitz C, Siren Al. An experimental protocol for mimicking pathomechanisms of traumatic brain injury in mice. Exp Transl Stroke Med 2012; 4: 1.

43) Woiciechowsky C, Schöning B, Didinger Gs, Stockhammer F, Volk Hd. Brain-IL-1β triggers astrogliosis through induction of IL-6: Inhibition by propranolol and IL-10. Med Sci Monit 2004; 10: 325-330.

44) Sairanen T, Carpen O, Karjalainen-Lindsberg Ml, Paetau A, Turpeinen U, Kaste M, Lindsberg Pj. Evolution of cerebral tumor necrosis factor-α production during human ischemic stroke. Stroke 2001; 32: 1750-1758.

45) Genovese T, Mazzon E, Crisafulli C, Esposito E, De Paola R, Muia C, Di Bella P, Meli R, Bramanti P, Cuzzocrea S. Combination of dexamethasone and etanercept reduces secondary damage in experimental spinal cord trauma. Neuroscience 2007; 150: 168-181.

46) Fix As, Ross Jf, Stitzel Sr, Switzer Rc. Integrated evaluation of central nervous system lesions: stains for neurons, astrocytes, and microglia reveal the spatial and temporal features of MK-801-induced neuronal necrosis in the rat cerebral cortex. Toxicol Pathol 1996; 24: 291-304.

47) Kita T, Liu L, Tanaka N, Kinoshita Y. The expression of tumor necrosis factor-α in the rat brain after fluid percussive injury. Int J Legal Med 1997; 110: 305-311.

48) Herrera Dg, Maysinger D, Almazan G, Funnel R, Cuello Ac. Analysis of c-Fos and glial fibrillary acidic protein (GFAP) expression following topical application of potassium chloride (KCl) to the brain surface. Brain Res 1998; 784: 71-81.

49) Oshima T, Lee S, Sato A, Oda S, Hirasawa H, Yamashita T. Tnf-α contributes to axonal sprouting and functional recovery following traumatic brain injury. Brain Res 2009; 1290: 102-110.

50) Pettigrew Lc, Kindy Ms, Scheff S, Springer Je, Kryscio Rj, Li Y, Grass Ds. Focal cerebral ischemia in the TNF alpha-transgenic rat. J Neuroinflamm 2008; 5: 47.

51) Mao M, Hua Y, Jiang X, Li L, Zhang L, Mu D. Expression of tumor necrosis factor α and neuronal apoptosis in the developing rat brain after neonatal stroke. Neurosci Lett 2006; 403: 227-232.

52) Hang Ch, Shi Jx, Tian J, Li Js, Wu W, Yin Hx. Effect of systemic LPS injection on cortical NF-κB activity and inflammatory response following traumatic brain injury in rats. Brain Res 2004; 1026: 23-32.

53) Campbell Sj, Jiang Y, Davis Ae, Farrands R, Holbrook J, Leppert D, Anthony Dc. Immunomodulatory effects of etanercept in a model of brain injury act through attenuation of the acute-phase response. J Neurochem 2007; 103: 2245-2255.

54) Tweedie D, Sambamurti K, Greig Nh. TNF-alpha inhibition as a treatment strategy for neurodegenerative disorders: New drug candidates and targets. Curr Alzheimer Res 2007; 4: 378-385.

55) Lopez Ne, Krzyzaniak Mj, Blow C, Putnam J, Ortiz-Pomales Y, Hageny Am, Eliceiri B, Coimbra R, Bansal V. Ghrelin prevents disruption of the blood-brain barrier after traumatic brain injury. J Neurotrauma 2012; 29: 385-393.

56) Lopez Ne, Krzyzaniak Mj, Costantini Tw, Putnam J, Hageny Am, Eliceiri B, Coimbra R, Bansal V. Vagal nerve stimulation decreases blood-brain barrier disruption after traumatic brain injury. J Trauma Acute Care Surg 2012; 72: 1562-1566.

57) Shlosberg D, Benifla M, Kaufer D, Friedman A. Blood-brain barrier breakdown as a therapeutic target in traumatic brain injury. Nat Rev Neurol 2010; 6: 393-403.

58) Marchand F, Tsantoulas C, Singh D, Grist J, Clark Ak, Bradbury Ej, Mahon Sb. Effects of etanercept and minocycline in a rat model of spinal cord injury. Eur J Pain 2009; 13: 673-681.

59) Wong Yw, Tam S, So Kf, Chen Jyh, Cheng Ws, Luk Kdk, Tang Sw, Young W. A three-month, open-label, single-arm trial evaluating the safety and pharmacokinetics of oral lithium in patients with chronic spinal cord injury. Spinal Cord 2011; 49: 94-98.