

Ultrastructural Changes in Tracheobronchial Epithelia Following Experimental Traumatic Brain Injury in Rats: Protective Effect of Erythropoietin

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- Background:** We aimed to demonstrate the time dependent ultrastructural changes in tracheobronchial epithelia after traumatic brain injury. And also, protective effect of erythropoietin was demonstrated.
- Methods:** We used 56 Wistar-Albino female rats weighing 170 to 200 g. The rats were allocated into 7 groups. First group was the control. The second underwent craniotomy without trauma. The third, fourth, and fifth groups were respectively 2-, 8-, and 24-hour trauma groups. The sixth and seventh groups were respectively treatment (erythropoietin, 1,000 IU/kg) and vehicle (0,4 ml/rat) groups. Weight-drop method was used for achieving head trauma. Samples were obtained from both trachea and main bronchi. Modified electron microscopic scoring model was used to reveal the ultrastructural changes in both trauma and treatment groups.
- Results:** There was no statistical difference between control and sham groups ($p > 0.05$). Scores of all trauma groups were significantly different from the controls ($p < 0.05$). Trauma produced obvious gradual damage on ultrastructure of the tracheobronchial epithelia. Erythropoietin decreased tracheobronchial scores after traumatic brain injury in significant levels. Erythropoietin attenuated ultrastructural scores for each organelle in significant levels ($p < 0.05$ for each organelle).
- Conclusions:** The data suggested that ultrastructural damage is obvious at 2 hours deteriorating with time. Erythropoietin protects epithelia against damage after traumatic brain injury. Pharmaceutical lung preservation may help gaining efficacious donor lungs in brain death. But, further time dependent experiments are needed to determine the liability of the donor lung after traumatic brain injury. This fact is to be known for achieving higher graft survival rates. *J Heart Lung Transplant* 2004;23:1423-9. Copyright © 2004 by the International Society for Heart and Lung Transplantation.

Airways are separated into two main parts, the conducting airways and the gas exchange region distal to the terminal bronchioles.¹

Airway epithelium is an absorptive and secretory type of epithelium.² The epithelium of the trachea consists of pseudo-stratified ciliated columnar epithelium with interspersed goblet cells.³

The mucociliary escalator of the lungs is an important protective transport system by means of which inhaled particles and microorganisms are removed from the tracheobronchial system.⁴

Lung mucociliary clearance is influenced by several factors, including the integrity of the ciliated epithelium and the thickness and physical properties of the periciliary or mucous layer.⁵

Traumatic brain injury (TBI) represents one of the most important causes of death and disability in modern society.⁶ Acute lung injury (ALI) is common in comatose victims with an isolated TBI and is associated with an increased risk of death or a severe neurologic morbidity.⁷ Mucociliary clearance has been reported to be impaired under mechanical ventilation.⁸

This pathologic process of lung leads to failure of brain oxygenation and increase the neurogenic injury. This further damage increases pathologic lung changes. Lung damage may result from free radicals coming out after central nervous system (CNS) injury,⁹ massive sympathetic discharge,¹⁰⁻¹⁵ and impairment of mucociliary activity.⁵ These pathologic pathways may lead to respiratory distress and ALI. Therefore, in case of donation, lower graft survival rates could only be achieved.

Injurious effects may be prevented using antioxidants such as erythropoietin (EPO). EPO has protective ef-

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fects against the damage, which appears following TBI. It has been reported that erythropoietin is a potent free radical scavenger decreasing oxidant injury.¹⁶ EPO is an endogenous cytokine with anti-apoptotic, anti-inflammatory, and neurotrophic properties.¹⁷ The neurotrophic and neuroprotective function of EPO in different conditions of neuronal damage, such as hypoxia, cerebral ischemia, and sub-arachnoid hemorrhage have been postulated.¹⁸

The aim of the current study was to demonstrate the ultrastructural pathologic findings following TBI in tracheobronchial epithelia in adult rats by a modified scoring model.¹⁹ And also, the protective effect of EPO on tracheobronchial epithelia against TBI induced damage was scored ultrastructurally.

MATERIAL AND METHODS

All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86 to 23, revised 1985).

The local Institutional Animal Care Committee approved protocols used in this study. The rats were randomly allocated into seven groups

Group 1

Group 1 (G1) is the control group ($n = 8$). Tissue samples were obtained immediately after thoracotomy and no head surgery was performed.

Group 2

Group 2 (G2) is the sham operated group ($n = 8$). Scalp was closed after craniotomy and no trauma was induced. Tissue samples were obtained 24 hours after surgical interventions.

Group 3

Group 3 (G3) is the 2-hr trauma group ($n = 8$). Traumatic brain injury of 140 g-cm was produced. Tissue samples were obtained 2 hours after trauma.

Group 4

Group 4 (G4) is the 8-hr trauma group ($n = 8$). Traumatic brain injury of 140 g-cm was produced. Tissue samples were obtained 8 hours after trauma.

Group 5

Group 5 (G5) is the 24-hr trauma group ($n = 8$). Traumatic brain injury of 140 g-cm was produced. Tissue samples were obtained 24 hours after trauma.

Group 6

Group 6 (G6) is the EPO group ($n = 8$). TBI of 140 g-cm was produced. R-Hu-EPO in a dose of 1,000 IU/kg (Eprex, Cilag AG, Zug, Switzerland) was administered intraperitoneally immediately after TBI. Tissue samples were obtained 24 hr after trauma.

Group 7

Group 7 (G7) is the albumin (vehicle) group ($n = 8$). TBI of 140 g-cm was produced. Vehicle solution (0.4 ml that contains 2.50 mg human serum albumin, 5.84 mg sodium chloride, 1.164 mg sodium phosphate monobasic dehydrate, and 2.225 mg sodium phosphate dibasic dehydrate per ml) was administered intraperitoneally immediately after TBI. We obtained tissue samples 24 hours after trauma.

Surgical Procedure

The surgical procedure was performed under general anesthesia induced by 10-mg/kg xylazine intramuscularly (Bayer, Istanbul, Turkey) and 60-mg/kg ketamine hydrochloride (Parke Davis, Istanbul, Turkey). Fifty-six female Wistar-Albino rats, weighing 170 to 200 g were used. Rats were placed in prone position. Following mid-line longitudinal incision, scalp was dissected over cranium and retracted laterally. Coronal and sagittal sutures were observed. Right frontoparietal craniectomies were carried out lateral to the sagittal suture by dental drill system. The dura was exposed and left intact. 140 g-cm impact traumas were produced by the method of Allen. Rats were injured by a stainless steel rod (5-mm diameter, weighing 140 g) weight dropped vertically through a calibrated tube from a height of 10 cm onto the exposed dura. Scalp was sutured with silk sutures.

Obtaining samples from trachea and bronchi. In 2, 8, and 24 hr after trauma groups, and 24 hr after sham operation and treatment groups, rats were re-anesthetized with the combination of ketamine and xylazine. Rats were placed supine on operating table. Mid-line sternotomy and bilateral thoracotomy was performed. The systemic circulation was perfused with 0.9% NaCl. Samples for electron microscopy were obtained from trachea and main bronchi. Then rats were killed with decapitation under general anesthesia. Samples were collected in randomly numbered containers and given to the blinded observers. After evaluating the numbered tissues, results were collected in the appropriate group lists.

Transmission Electron Microscopy

The specimens were fixed in 2.5% glutaraldehyde for 24 hours, washed in phosphate buffer (pH: 7.4), post-fixed in 1% osmium tetroxide in phosphate buffer (pH:

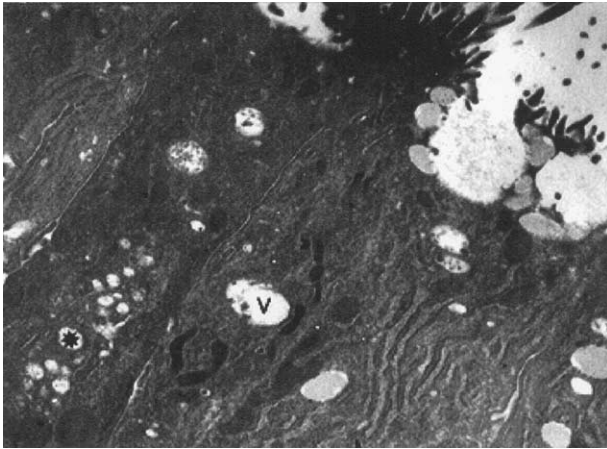


Figure 1. Electron micrograph illustrating ultrastructural pathologic findings in the 2-hour trauma group (G3) (original magnification $\times 7,500$).

7.4), and dehydrated in increasing concentrations of alcohol. Then the tissues were washed with propylene oxide and embedded in epoxy-resin embedding media. Semi-thin sections about 2 μm in thickness and ultra thin sections about 60 nm in thickness were cut with a glass knife on an LKB-Nova (Sweden) ultra microtome. Semi-thin sections were stained with methylene blue and examined by a Nikon Optiphot (Japan) light microscope. Ultra thin sections were collected on copper grids, stained with uranyl acetate and lead citrate, and examined with a Joel JEM 1,200 EX (Japan) transmission electron microscope. A modified injury-scoring model was used to evaluate the ultrastructural changes following TBI.¹⁹

Tracheobronchial Scores

Nucleus (N). Scores were determined as: 0 = normal; 1 = disintegrated chromatin (margination, clumping); 2 = increased heterochromatin; 3 = degenerated nuclei.

Granulated endoplasmic reticulum (GER). Scores were determined as: 0 = normal; 1 = dilated; 2 = corrupted lamellar arrangement; 3 = broken.

Smooth endoplasmic reticulum (SER). Scores were determined as: 0 = normal; 1 = dilated in places; 2 = existing vacuoles; 3 = fields of broad degeneration + myelin figures.

Mitochondria (M). Scores were determined as: 0 = normal; 1 = clear cristae; 2 = edematous; 3 = accumulation of amorphous material.

Statistical Analysis

All the data collected from the experiment was coded, recorded, and analyzed by using SPSS 10.0.1 for Windows (Chicago, IL). Kruskal-Vallis variance test for

non-parametric data were used for comparing differences between groups. When analysis of variance revealed a significant difference, the post-hoc multiple comparison test was applied to demonstrate the differences in the groups. In each test, the data were expressed as the mean value \pm standard error (SE) and $p < 0.05$ was accepted as statistically significant.

RESULTS

The ultrastructural findings were the same in both trachea and the bronchi epithelia. In the following text, the changes in the tracheal epithelia were described in detail.

Transmission Electron Microscopic (Scoring)

G1–G2. In the transmission electron microscopic (TEM) examination of trachea, no ultrastructural pathology was observed.

G3. In the trachea epithelium, vacuole formation was present in the smooth endoplasmic reticulum and mitochondrial swelling was also observed. Additionally, an intercellular edema was present in the connective tissue of the trachea (Figure 1).

G4. The same ultrastructural findings were observed in the trachea, however, vacuoles in this group were much more larger than G3 group. Additionally, a mild edema was present around the epithelial cells (Figure 2).

G5. The same ultrastructural findings, observed in G4 group, were present. However, the amount of ultrastructural pathology was much more prominent than the other groups. And also, the amount of edema around the epithelial cells was more prominent than the one in G4 group (Figure 3).

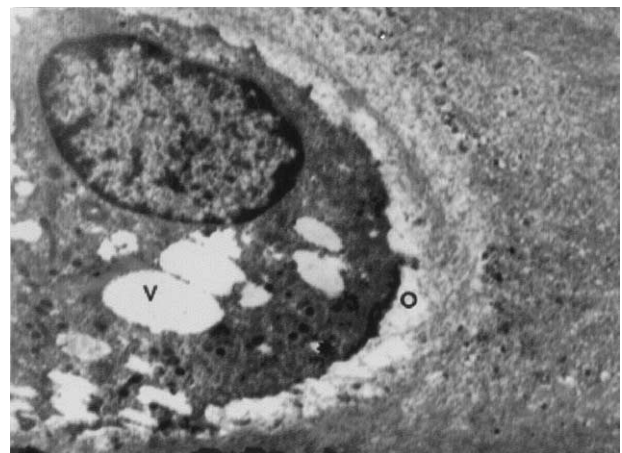


Figure 2. Electron micrograph revealing ultrastructural pathologic findings in the 8-hour trauma group (G4) (original magnification $\times 7,500$).

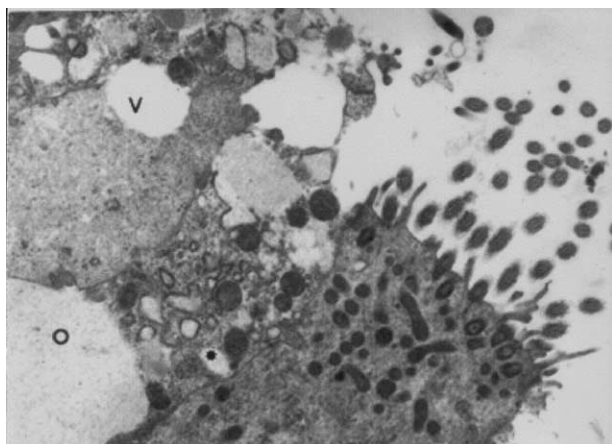


Figure 3. Electron micrograph demonstrating ultrastructural pathologic findings in the 24-hour trauma group (G5) (original magnification $\times 7,500$).

G6. In the epithelium of trachea, vacuole formation was present in the smooth endoplasmic reticulum and mitochondrial swelling was also observed. These ultrastructural pathologic findings were also seen in the cytoplasm of smooth muscle cells. However, the degree of these pathologic changes was lesser than G3, G4, and G5 groups (Figure 4).

G7. Swelling of the mitochondria and vacuole formation in the smooth endoplasmic reticulum were observed in all the layers of trachea and the amount of these pathologic changes were much more prominent and severe than G6 group ultrastructurally.

TEM and Statistical Results

General tracheobronchial scores. There was no statistically difference between control and sham operated

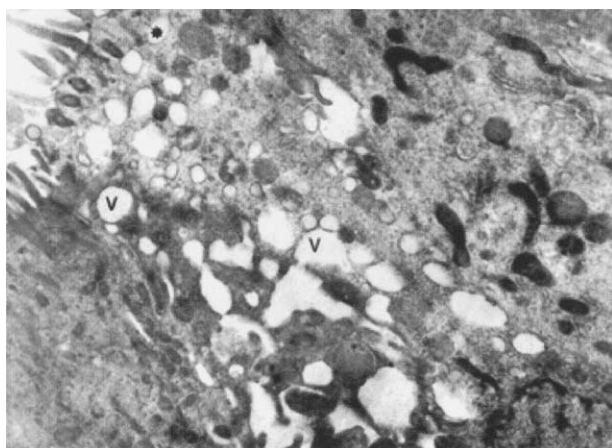


Figure 4. Electron micrograph illustrating no significant pathologic findings in the group of 24-hour trauma group given erythropoietin meaning protective effect of the erythropoietin (G6) (original magnification $\times 7,500$).

Table 1. TBS Scores After Both TBI and EPO Treatment

Group	EM score (mean \pm SE)
G1 (Control)	0.08 \pm 0.01
G2 (Sham)	0.10 \pm 0.01
G3 (Trauma 2 hrs)	0.75 \pm 0.05
G4 (Trauma 8 hrs)	0.88 \pm 0.05
G5 (Trauma 24 hrs)	1.04 \pm 0.06
G6 (EPO)	0.74 \pm 0.05
G7 (vehicle)	0.99 \pm 0.06

There was statistically difference between G3, G4, and G5 groups regarding TBS ($p < 0.05$). Trauma produced obvious gradual damage on ultrastructure of the tracheobronchial epithelia in time dependent manner. There was no statistically significant difference between G3 and G6 groups ($p > 0.05$). EPO decreased TBS in significant levels comparing to G4 and G5 groups. Vehicle had no protective effect on tracheobronchial epithelia. TBS, tracheobronchial scores; TBI, traumatic brain injury; EPO, erythropoietin.

animals ($p > 0.05$). Scores of all trauma groups were significantly different from the controls ($p < 0.05$). There was statistically difference between G3, G4, and G5 groups regarding general score ($p < 0.05$). Trauma produced obvious gradual damage on ultrastructure of the tracheobronchial epithelia in time dependent manner. All trauma groups were different from each other in significant levels ($p < 0.05$ for each trauma group). There was no statistically difference between G5 and G7 groups ($p > 0.05$). Vehicle solution has no protective effect on tracheobronchial epithelia. There was no statistically significant difference between G7 and G3 groups ($p > 0.05$). EPO decreased TBS scores after TBI in significant levels. Results of TBS were shown in Table 1.

The effect of TBI was evaluated for each organelle, including nucleus, mitochondria, SER, and GER depending on ultrastructural scores. There was no statistically significant damage in GER and nucleus for each group ($p > 0.05$). GER and nucleus were resistant to damage following TBI. EPO attenuated ultrastructural scores for each organelle, except GER and nucleus in significant levels ($p < 0.05$ for each organelle). EPO had a definite protective effect for smooth endoplasmic reticulum and mitochondrion.

DISCUSSION

Our results indicated that TBI caused pathologic changes in lungs that TBS model clearly documented. EPO moderately prevented these damages and prevented pathologic changes in tracheobronchial epithelia. Albumin had no protective effect. There was no significant difference between tracheal and bronchial epithelia according to the degree of damage. The smooth endoplasmic reticulum and the mitochondrion were mainly affected from TBI, whereas the granular endoplasmic reticulum and the nucleus were both resistant to the TBI.

TBI may cause lung damage through various mechanisms mentioned in the introduction section. After TBI, loss of cell ion homeostasis happens in the brain. The

Ca²⁺ ion and lactic acid levels increase immediately afterwards. And then, neurotransmitters such as dopamine, noradrenalin and glutamate, and NO and Fe²⁺ increase. Ultimately, free radicals come out and cause structural changes, glial injury, necrosis, and apoptosis. The result is neuronal damage.⁹ Free radical overload leads to the damage of many cellular components, including proteins, DNA, and phospholipids, thus causing denaturation and cellular dysfunction. In addition, free radical attack on membrane lipids can produce substances that can be cytotoxic or have biologic activity leading to changes in membrane fluidity and integrity.²⁰

As the trachea and bronchia are the conducting airways, their main function, the mucociliary activity, is very important to achieve keeping the respiratory tract clear of particles and microorganisms.

The efficiency of the mucociliary system depends not only on the integrity of the epithelium and ciliary activity but also on the amount of mucus, the depth of the periciliary layer and the physical properties.^{5,21,22}

Lung mucociliary clearance is influenced by several factors.⁵ Mucociliary clearance has been reported to be impaired under mechanical ventilation.⁸

In the present study, trauma produced obvious gradual deteriorating damage on ultrastructure of the tracheobronchial epithelia in time dependent manner. All trauma groups were different from each other in significant levels ($p < 0.05$ for each trauma group).

Smooth endoplasmic reticulum is involved in the synthesis of lipids and carbohydrates. And it has an important role in Ca²⁺ storage along with other cellular processes.²³ Adenosine A³ receptor agonist enhances airway mucociliary clearance probably through Ca²⁺ mediated stimulation of ciliary motility of airway epithelium.²⁴

Mitochondria are responsible for energy production, Krebs cycle. Various reactions in the cell can either use energy or produce it.²³

Ciliary motility is dependent on the function of the motor protein dynein, sharing attributes of adenosine triphosphatase (ATPases). Ciliary beat frequency is further dependent on medium conditions. The amount of immunohistochemically detectable ATPase sub-unit positive cells strongly correlates with ciliary motility *in vitro*.²⁵

As a result, the energy production process in the mitochondria and the Ca²⁺ storage role of the smooth endoplasmic reticulum may probably be prevented to process following TBI. The worse impact of free radicals and sympathetic discharge on the ultrastructure of the tracheobronchial epithelia may result in some degree losing the ability of motility of the respiratory cilia concluding in disturbance of the protective transport system. As a result, inhaled particles and microorganisms collect in the retained

respiratory secretions leading to atelectasis and recurrent infections. This prevents the vital oxygenation function of the lung to occur. In case of donation of this lung, lower graft survival rates should merely be expected.

As mentioned in the literature, there is a strict shortage of organ donation worldwide. The lower graft survival rates are associated with the race, sex, age, and cause of death.^{26,27} The lung transplantation donors are the TBI patients in almost half of the cases.^{28,29} The worthy lungs to be harvested from TBI patients should be protected as much as possible utilizing all effective methods.

Although the events leading to TBI may not be prevented, a more aggressive response to its treatment may perhaps improve the degree of recovery of brain.¹¹ Some different pharmaceutical agents have been used to prevent these deteriorating events whether experimentally and clinically. Treatment with antioxidants may act to prevent propagation of tissue damage and improve both the survival and neurologic outcome. Antioxidants are exogenous (natural or synthetic) or endogenous compounds acting in several ways including removal of O₂, scavenging radical oxygen species (ROS) or their precursors, inhibiting ROS formation and binding metal ions needed for catalysis of ROS generation.⁹

As with other neuroprotectant, to achieve high efficacy, anti-oxidants must be given as early as possible. The therapeutic window for successful attenuation of an infarct volume was demonstrated to be 3 to 4 hours in rats.⁹ In the treatment group, we administered EPO intraperitoneally immediately after TBI.

EPO is an endogenous cytokine with anti-apoptotic, anti-inflammatory, and neurotrophic properties. Apart from being produced by the kidney, liver, and spleen in response to hypoxia, EPO is highly expressed in the brain during development and after neuropathological insults such as hypoxia, cerebral ischemia, and subarachnoid hemorrhage.^{17,18,30,31} Pre-clinical findings suggest that EPO may have therapeutic potential for stroke, head trauma, and epilepsy.^{17,18,32} It has been shown that EPO is a potent free radical scavenger decreasing oxidant injury.^{16,33}

On the other hand, erythropoietin receptors (EPO-R) have been demonstrated on several non-hematopoietic cell types in animal models and in cell culture. EPO-R is expressed on many cell types during early fetal development.³⁴

It has been reported that N-methyl-D-aspartate receptors (NMDA) exist in the lung, that their activation triggers acute injury, and that, as in toxicity to central neurons, this injury is associated with stimulation of nitric oxide (NO) synthesis, and can be attenuated by inhibition of this synthesis.³⁵ Another effect mechanism

defined for EPO may be inhibiting NMDA receptors and reducing the NO-mediated formation of free radicals or its direct free radical scavenging effect on lung tissue. Excessive generation of NO causes mitochondrial dysfunction. NO and O_2^- react and result in large amounts of peroxynitrite ($ONOO^-$) generation.³⁶ Peroxynitrite causes DNA damage via oxidative reactions.³⁷ It has been shown that EPO was neuroprotective against NO-induced neuronal cell death. EPO may increase the activities of anti-oxidant enzymes such as superoxide dismutase, glutathione peroxidase, and catalase in neurons.³³

In our study, TBS results supported the data concluded by EPO study. EPO protected the tracheobronchial epithelia against the damage produced by TBI probably using the mechanisms in combination as described above. This protective effect of EPO may result in better recovery of TBI patients. Therefore achieving higher lung donation rates in brain death patients could strongly be expected.

At the same time, antioxidant therapy could possibly offer protection to other solid organ transplants. There are several mechanism described in the literature. As, induction of an anti-oxidative stress protein and inhibition of proinflammatory cytokines³⁸; decreasing activation of nuclear factor-kappa B (NF-kappa B), an important redox-sensitive transcription factor necessary for iNOS gene expression³⁹; inducing the expression of the protective enzyme heme oxygenase-1 in cultured endothelial cells by an oxidative mechanism.⁴⁰

In conclusion, here we tried mainly to bring up the pathologic changes ultrastructurally and the protective effect of EPO against tracheobronchial injury following isolated TBI in rats. The probable hindered mucociliary activity as a result of TBI may progress to atelectasis and recurrent infections and in conclusion may lead to unsuitable lung donations in transplantation. This study evidently reflected the pathologic findings following TBI in rats. The aim of preserving tracheobronchial epithelia is to prevent injury after TBI, therefore, increasing the chance of lung being fit for donation. Other studies are required to set the exact damage mechanisms in mucociliary activity and the mucus secreting goblet cells, if ever. Further experiments are also needed to determine the exact dose, mechanism and timing of EPO treatment to help in achieving optimal lung donation after TBI in animal lung transplantation models. Physicians should notice of this fact for providing higher graft survival rates in the world of donor shortage.

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