*Original Article*

# Apoptosis-Related Gene Bcl-2 in Lung Tissue After Experimental Traumatic Brain Injury in Rats

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*Background:* **We have recently shown that experimental traumatic brain injury resulted in ultra structural damage in lung tissue. The main objective of the current study was to investigate in a rat model of brain injury whether expression of Bcl-2 gene and lipid peroxidation levels in the lung tissue after traumatic brain injury were affected by methylprednisolone sodium succinate (MPSS) treatment.**

*Methods:* **Fifty-six Wistar-Albino female rats weighing 180–220 g were used, which were allocated into seven groups. A weight-drop method was used to achieve head trauma. Real time quantitative PCR analyses for Bcl-2 gene expression and measurement of the levels of lipid peroxidation were carried out. All the data was analyzed by using SPSS 11.5 for Windows.**

*Results:* **Mean Bcl-2 expression in the methylprednisolone group was considerably higher compared to that of all the other groups ($p < .05$). Mean lipid peroxidation levels were significantly higher in the trauma group and notably lower in the methylprednisolone group ($p < .01$).**

*Conclusions:* **The oxidative stress imposed on lung tissue, as seen by high levels of lipid peroxidation, after brain injury was significantly attenuated by MPSS treatment. MPSS treatment following brain injury also augmented putative anti-apoptotic Bcl-2 gene expression in lung tissue. Further studies are required to determine the full range and lower limits of effective MPSS dose. More importantly the optimal efficacy according to the timing of MPSS treatment after brain injury needs to be determined for impact on more diverse markers of cell inflammation, apoptosis and injury.**

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**Keywords. Trauma; Blunt; Animal model; Brain; Lung; Apoptosis; Lung transplantation**

## Introduction

As we have thoroughly described in a previous study,[1] the principal pathophysiological processes in acute central nervous system injury are enormously complex and may include pathological permeability of blood brain barrier, increased neurotransmitters, free radical-mediated toxicity, and neurogenic pulmonary oedema.

Cell death or survival following central nervous system injury may be a consequence of alterations in the intracellular ratio of death and survival factors.[2] It was shown in rats that trauma triggers diffuse apoptotic neurodegeneration in the developing brain,[3] and increases the expression of Bcl-2 in brain neuron. A negative correlation is seen between Bcl-2 expression and neuronal apoptosis.[4]

Ischemic reperfusion injury may also lead to apoptotic cell death in lung tissue.[5] Increased Bcl-2 gene levels suggest that the apoptotic cascade after diffuse traumatic brain injury is a carefully controlled cellular homeostatic response. Pharmacological manipulation of this balance may offer a therapeutic approach for preventing cell death and improving outcome after diffuse traumatic brain injury.[6]

It is of paramount importance to achieve greater numbers of better quality donor lungs as donor organ

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shortage severely limits lung transplantation as a therapeutic option.[7] Causes of the low deceased donor rate appear to include variable management of severe brain injury.[8]

Induction of apoptosis of myeloid leukemic cells is possible by short-course high dose MPSS treatment.[9] Additionally, MPSS has also a protective effect on reperfusion injury using the anti-lipid peroxidation (LPO) activity,[10] and it has a biphasic effect on alveolar capillary integrity after elevated cerebrospinal fluid pressure.[11]

In the current study, we tested in an animal model whether expression of Bcl-2 gene and LPO levels in the lung tissue after traumatic brain injury was affected by MPSS treatment. We propose that MPSS is a therapeutic agent which might be useful achieving better quality donor lungs following brain death.

## Materials and Methods

### *Experimental Groups*

All animals received humane care in compliance with "the Guide for the Care and Use of Laboratory Animals" (National Institutes of Health, USA, Publication No. 85-23, revised 1996). The Animal Care Committee of the Ankara Hospital approved the protocols used in this study.

The rats' brains were injured using the weight-drop method.[12] Impact of 300 g cm brain injuries was produced in the T, MPSS, and V treatment groups (see below). Tissue samples were obtained 24 h after traumatic brain injury in all groups except control.

Following brain injury lung tissue specimens were collected in order to measure Bcl-2 gene expression and LPO levels.

Fifty-six female Wistar-Albino rats, weighing 180–220 g, were randomly allocated into seven groups.

C (control) ($n = 8$): Tissue samples immediately after thoracotomy; no head surgery.
Cmpss (control-MPSS) ($n = 8$): MPSS (MPSS = methylprednisolone sodium succinate); intraperitoneally-bolus (30 mg/kg), 24-h prior to thoracotomy.
S (sham operated) ($n = 8$): Scalp was closed after craniotomy; no trauma.
Smpss (sham-MPSS) ($n = 8$): MPSS; intraperitoneally-bolus (30 mg/kg), immediately after craniotomy.
T (trauma) ($n = 8$).
MPSS (MPSS) ($n = 8$): MPSS; intraperitoneally-bolus (30 mg/kg), instantly after trauma.
V (vehicle) ($n = 8$): Saline (0.9%); intraperitoneally-bolus (0.1 cc/rat), directly after trauma.

### *Surgical Procedure*

The surgical procedure was performed under general anaesthesia induced by intramuscular xylasine (Bayer, Istanbul, Turkey) (10 mg/kg) and ketamine hydrochloride (Parke Davis, Istanbul, Turkey) (60 mg/kg) injections. Rats each were placed in prone position. Right frontoparietal craniectomies were carried out lateral to the sagittal sinus by dental drill system. The dura was exposed and left intact. Trauma of 300 g cm impacts was produced by the weight-drop method.[12] Body temperature was continuously monitored with a rectal thermometer and maintained at 37 °C using a heating pad and an overhead lamp. Rats were not intubated. They were given free access to food and water.

### *Obtaining Samples from Lung Parenchyma*

Twenty-four hours after traumatic brain injury for all groups except the control group, rats were re-anaesthetized. Midline sternotomy and bilateral thoracotomy were performed. The systemic circulation was perfused with 0.9% NaCl. Then, rats were killed with decapitation under general anaesthesia. Samples were all obtained from the left pulmonary lobes. Samples for Bcl-2 expression were immediately frozen in liquid nitrogen and then stored at −80 °C. 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.

### *Bcl-2 Expression*

ISOLATION OF RNA AND SYNTHESIS OF CDNA. Total RNA of lung tissue was isolated using High pure RNA tissue kit (Roche Diagnostics, Germany). RNA integrity was electrophoretically verified by ethidium bromide staining and by OD 260 nm/OD 280 nm absorption ration >1.95. One μg of total RNA was used for cDNA synthesis using 1st Strand cDNA synthesis kit for RT-PCR (AMV) (Roche Diagnostics, Germany) according to the manufacturer's instructions.

QUANTITATIVE REAL TIME PCR ANALYSIS. Real time quantitative PCR analyses for Bcl-2 gene expression were performed as described previously[13] using a LightCycler™ instrument (Roche Diagnostics). Amplification was performed in 20 μl volumes, which included 2 μl cDNA, 4 nM of each primer, 2 nM of TaqMan probe and LightCycler™ DNA master hybridization master mix. The cycling parameters were 2 min at 95 °C for denaturation, 40 cycles of 15 s at 95 °C, 30 s at 60 °C for amplification and quantification respectively. With β-actin mRNA was quantified to adjust the amount of mRNA in each sample with β-actin probe and primer set. The upstream and downstream primer sequences were 5′ TCTTTAATGTCACGCACGATT and 5′ TCACCCACACTGTGCCCAT, respectively, and the TaqMan probe selected between the primers was fluorescence labelled at the 5′ end with 6-carboxyfluorescein (FAM) as the reporter dye and at the 3′ end with 6-carboxytetramethylrhodamine (TAMRA) as the quencer; 5′-FAM-ATCCTGCGTCTGGACCTGGCTTAMRA (Tibmolbiol, Germany).[14,15] Using the same PCR conditions as described above, the level of the housekeeping gene β-actin was also quantified so as to normalize Bcl-2 gene expression values of each sample. Relative expressions were calculated according to the mathematical model of Pfaffl MW based on the PCR efficiencies and the crossing points.[16]

### *Lipid Peroxidation Assay*

The samples were thoroughly cleansed of blood and were immediately frozen and stored in a −20 °C freezer for assays of malondialdehyde. The levels of LPO were measured as thiobarbituric acid-reactive material.[17] Using tetramethoxypropane as the standard, tissue LPO levels were calculated as nanomole per gram of wet tissue.

### *Statistical Analysis*

All the data collected from the experiment was coded, recorded, and analyzed by using SPSS 11.5 statistical software package for Windows. All the nominal data were expressed as the mean ± S.D. The shapiro-wilk test was used to normalize the measured values of the groups. The one-way analysis of variance (ANOVA) was used to compare the measured values among the groups. Tukey's honestly significant difference (Tukey-HSD) test was applied to determine the statistically significant differences between the groups, as post hoc. $p < .05$ was accepted as statistically significant.

## Results

Fig. 1 shows the mean gene expression of Bcl-2 gene across the treatment groups. One-way ANOVA and post hoc tests revealed that mean Bcl-2 expression of the group MPSS

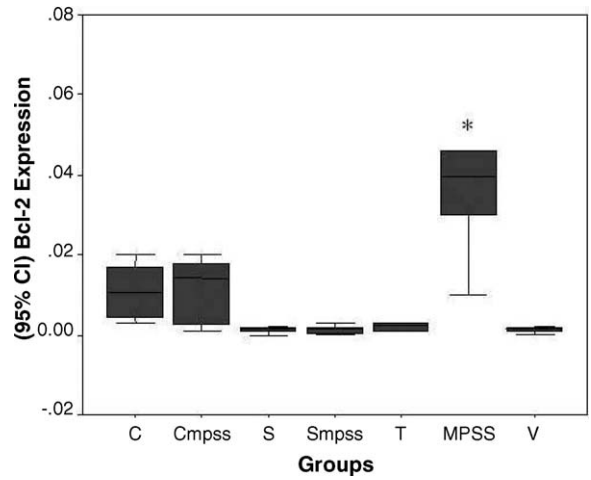


**Figure 1.** *Effect of traumatic brain injury on lung Bcl-2 expressions in the groups included. Graph shows mean (95% CI) lung tissue Bcl-2 expression levels after traumatic brain injury. Group MPSS has the highest level of the expression of Bcl-2 than the other groups (*). Each study group consisted of eight rats. Abbreviations: C: control group; Cmpss: control-methylprednisolone treatment group; S: sham-operated group; Smpss: sham-methylprednisolone treatment group; T: trauma group; MPSS: methylprednisolone sodium succinate treatment group; V: vehicle. TBI: traumatic brain injury; CI: confidence interval for mean; *: significant increase (p < .05).*

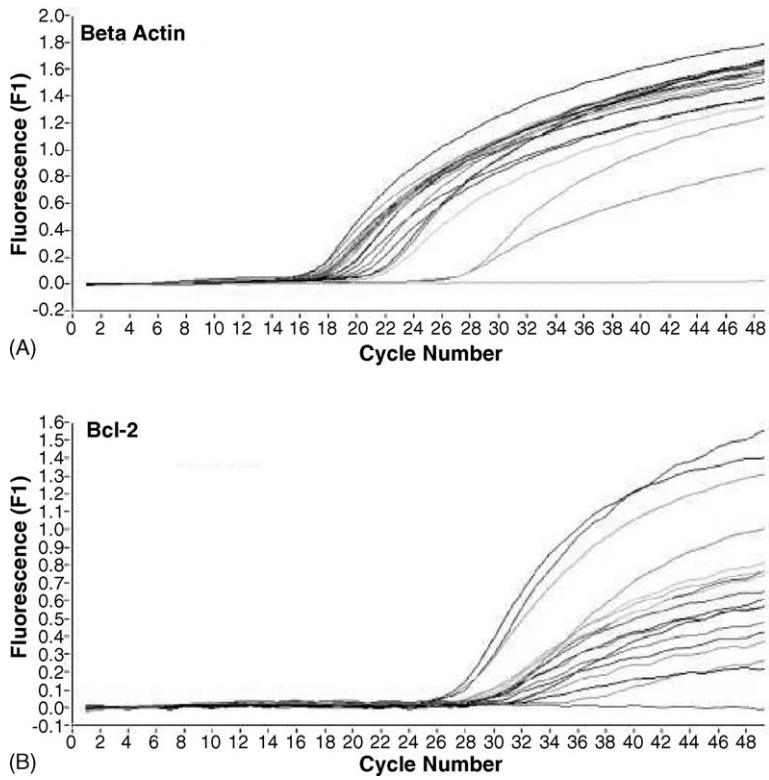


**Figure 2.** *Real-time quantitative PCR for gene expression analysis. cDNA from rat lung tissue samples were PCR amplified in the presence of TaqMan probes. Plots of fluorescence signals (X-axis) vs. cycle number (Y-axis) using each region primers/probes and cDNA samples are shown in diagrams [β-actin (A) and Bcl-2 (B) amplification curves]. The expressions of Bcl-2 gene were compared with β-actin expression of each sample. The negative controls without target cDNA show no signal increase in the diagrams. Abbreviations: PCR: polymerized chain reaction; cDNA: cellular DNA.*

**Table 1.** *Proportional Increase and/or Decrease in Bcl-2 Gene Expression Compared to the Control Group (C)*

| | | | 95% Confidence Interval | | | |
|---|---|---|---|---|---|---|
| Groups | *n* | Mean ± S.D. | Lower Bound | Upper Bound | Increase (%) | Decrease (%) |
| C | 8 | 0.0109 ± 0.067 | 0.0038 | 0.018 | 100 | 100 |
| Cmpss | 8 | 0.0115 ± 0.008 | 0.0031 | 0.20 | 105.50 | |
| S | 8 | 0.0013 ± 0.0007 | 0.0005 | 0.002 | | 11.93 |
| Smpss | 8 | 0.0014 ± 0.0011 | 0.00026 | 0.0026 | | 12.84 |
| T | 8 | 0.0020 ± 0.0009 | 0.0011 | 0.003 | | 18.35 |
| MPSS | 8 | 0.0391 ± 0.0198 | 0.018 | 0.06 | 358.71[a] | |
| V | 8 | 0.0019 ± 0.00164 | 0.00019 | 0.0036 | | 17.43 |
| Total | 56 | 0.0097 ± 0.015 | 0.005 | 0.0144 | | |

*Abbreviations*: C: control group; Cmpss: control-methylprednisolone treatment group; S: sham-operated group; Smpss: sham-methylprednisolone treatment group; T: trauma group; MPSS: methylprednisolone sodium succinate treatment group; V: vehicle.
[a] Take note that the only significant increase in Bcl-2 gene expression is determined in the treatment group MPSS.

was significantly higher compared to that of the other treatment groups ($p < 0.05$).

In Fig. 2, real-time quantitative PCR for gene expression analysis is shown. The expression of Bcl-2 gene was compared with β-actin expression of each sample. The negative controls without target cDNA show no signal increase in the diagrams.

Table 1 shows the proportional (%) increase and/or decrease in Bcl-2 mRNA expression compared to the control group (100%). Note that the only significant increase in Bcl-2 gene expression is in the treatment group MPSS.

Mean lung thiobarbituric acid-reactive substance levels are shown in Table 2. The control, control-MPSS, and sham-MPSS groups had significant differences than the trauma and vehicle groups ($p < .01$). The trauma group had the highest levels for LPO than the other groups ($p < .01$) except vehicle group. MPSS significantly lowered the LPO levels compared to that of the trauma and vehicle groups ($p < .05$).

## Discussion

In the present study, our primary goal was to examine the expression of Bcl-2 gene in the lung tissue occurring after brain injury. The secondary aim was to test whether MPSS treatment after brain injury altered the expression of Bcl-2 gene in the lung tissue. Our data shows that MPSS treatment after brain trauma augmented Bcl-2 gene expression and reduced LPO in lung tissue.

Brain trauma is a major cause of morbidity and mortality, both in adult and paediatric populations. Much of the functional deficit derives from delayed cell death resulting from induction of neurotoxic factors that overwhelm endogenous neuroprotective responses.[18] Brain injured patients have an increased risk of extra cerebral organ failure, mainly pulmonary dysfunction.[19]

Increasing evidence suggests that pulmonary dysfunction resulting from acute oxygen toxicity is at least in part due to the injury and death of lung cells. Studies using morphological and biochemical analyses revealed that hyperoxia-induced pulmonary cell death is multimodal, involving not only necrosis, but also apoptosis. A correlative relationship between the severity of hyperoxic acute lung injury and increased apoptosis has been supported by numerous studies in a variety of animal models. Cell death and lung injury are associated with increased expression of several apoptotic regulatory proteins such as p53 and Bcl-2, and DNA damage-induced proteins.[20]

Besides, exposure to hyperoxia may result in lung injury that is characterized by apoptosis and necrosis of the alveolar epithelium and endothelium. Several groups of investigators have demonstrated that cultured cells undergo apoptosis following exposure to hyperoxia.[21]

**Table 2.** *Effect of TBI on Lung TBARS Levels in the Groups Included (LPO Activity (nmol/g Wet Tissue))*

| | | | | 95% Confidence Interval for Mean | | | | |
|---|---|---|---|---|---|---|---|---|
| Groups | Mean | S.D. | S.E. | Lower Bound | Upper Bound | Min | Max | *p*-value |
| C | 40.31 | 10.82 | 3.82 | 31.27 | 49.35 | 25.73 | 55.46 | |
| Cmpss | 39.52 | 9.12 | 3.22 | 31.90 | 47.14 | 26.30 | 50.89 | 1.00 |
| S | 41.03 | 10.52 | 3.72 | 32.23 | 49.82 | 29.73 | 56.60 | 1.00 |
| Smpss | 39.09 | 9.26 | 3.27 | 31.35 | 46.84 | 28.59 | 52.60 | 1.00 |
| *T* | *61.75* | *12.33* | *4.36* | *51.45* | *72.06* | *46.89* | *78.91* | 0.003 |
| *MPSS* | *43.02* | *12.34* | *4.36* | *32.71* | *53.34* | *29.16* | *59.46* | 0.998 |
| V | 59.39 | 7.63 | 2.70 | 53.01 | 65.77 | 52.03 | 73.76 | 0.01 |

Lipid peroxidation levels were significantly higher in the trauma group (T) and the vehicle group (V) compared to that of the control group (C) ($p < .01$). The levels were considerably lower in the methylprednisolone group (MPSS) compared to that of the trauma (T) and the vehicle (V) groups ($p < .05$). Take note that lipid peroxidation levels of Smpss, S, and Cmpss were no significantly different than that of the control group ($p < .05$). Each study group consisted of eight rats. *Abbreviations*: C: control group; Cmpss: control-methylprednisolone treatment group; S: sham-operated group; Smpss: sham-methylprednisolone treatment group; T: trauma group; MPSS: methylprednisolone sodium succinate treatment group; V: vehicle. TBI: traumatic brain injury; TBARS: thiobarbituric acid-reactive substance; LPO: lipid peroxidation.

Although, it represents a physiologic clearance mechanism in human tissues,[22] apoptosis of neurons and glia contribute to the overall pathology of traumatic brain injury in both humans and animals. In both head-injured humans and following experimental brain injury, apoptotic cells have been observed alongside degenerating cells exhibiting classic necrotic morphology. Neurons undergoing apoptosis have been identified within contusions in the acute post-traumatic period and in regions remote from the site of impact in the days and weeks after trauma.[23]

Yoneda et al. have shown that oxidant injury induces Bcl-2 genes that are involved in the regulation of apoptosis.[24] Some other investigators demonstrated that ischemic reperfusion injury may also induce human lung cell apoptosis.[5] It has been extensively shown that over expression of regulating gene Bcl-2 has protective effect against apoptosis in different organs such as lungs,[5] intestine,[25] and liver.[26]

Limiting apoptosis throughout injury is related to enhanced survival and organ function.[27] According to the above mentioned reports, it appears that induction of the Bcl-2 gene expression may protect lung tissue after traumatic brain injury. It has been shown that even "ischemic preconditioning" may reduce apoptosis of human lung cells in vivo by up regulating Bcl-2 protein expression.[5] Thus the use of a therapeutic agent such as MPSS, as per the current study appears a useful approach for protecting lung tissue from the subsequent effects of brain injury and death.

There is no single intervention or agent that has emerged as being beneficial in severely brain injured patients.[28] Several such agents of widely varying chemical structures have been investigated as therapeutic agents for acute central nervous system injury. Although a few of the antioxidants showed some efficacy in animal models or in small clinical studies, these findings have not been supported in comprehensive, controlled trials in patients.[29,30]

Treatment with antioxidants and free radical scavengers such as antioxidant *N*-acetylcysteine[31] and pentoxyfilline[32] may hypothetically act to avert propagation of tissue damage and improve both the survival and neurological outcome.[29]

Glucocorticoids may inhibit or promote apoptosis in various cell types.[33] Dexamethasone enhanced the neurotrophin-3 expression after traumatic brain injury, indicating that post-traumatic neuroprotection in the hippocampus is at least partially mediated by neurotrophin-3 and thus can be modulated by dexamethasone treatment.[34] Modulation of mineralocorticoid receptor gene expression may therefore be an important target for reduction of brain injury in conditions caused by cerebral ischemia including brain damage following cardiac arrest and stroke.[35] On the other hand, in another study it was stated that spironolactone and mifepristone decreased basal Bcl-2 messenger RNA levels in CA1 and dentate gyrus. Mifepristone modulation of Bcl-2, Bax, or p53 messenger RNA expression does not predict neuronal viability.[36]

To address the importance of modulating the Bcl-2 gene expression in lung tissue after the traumatic brain injury, we studied MPSS in the current experimental head trauma model. It is known that MPSS has a protective effect on reperfusion injury using the anti-LPO activity.[10] We have clearly shown that MPSS had significantly anti-LPO activity which means reduction in membrane lipolysis in the lung tissue after head trauma.

Additionally, MPSS has been shown to have a biphasic effect on alveolar capillary integrity after elevated cerebrospinal fluid pressure,[11] whereby low dose MPSS minimized the extent of lung haemorrhage, pulmonary capillary leakage, and loss of lung compliance. In contrast, MPSS high dose accelerated tissue haemorrhage and compliance loss, even though pulmonary capillary permeability was maintained near base line rates.[11] However, guidelines for the management of acute spinal cord injury still recommend MPSS.[37]

The efficacy of glucocorticoids in the treatment of multiple sclerosis may involve the induction of T cell apoptosis. Glucocorticoids have two different effects on the vulnerability of human antigen-specific T cells: (1) steroids induce T cell apoptosis in a CD95-independent, but caspase-dependent manner; (2) steroids protect T cells from CD95-m-*ediated apoptosis, which is also caspase-dependent. An increase in Bcl-2 expression is observed upon incubation with steroids;[38] in contrast, Uckan et al. informed that the changes in Bcl-2 expression after high dose MPSS was not significant in acute lymphoblastic leukaemia.[39]

In conclusion, this study provides evidence for the ability of potentially therapeutic agent, MPSS, to induce the expression of Bcl-2 gene in the lung tissue after traumatic brain injury. Moreover, MPSS, an easily available and cheap agent, might provide a new avenue for therapy to minimise lung damage after brain injury.

Further studies are required to test for the efficacy of dose and time of MPSS treatment post-brain injury. In addition, animal studies that directly testing MPSS treatment effects on measures of lung function plus more diverse markers of inflammation and cell injury are needed. Such proposed studies are necessary to determine whether higher graft survival rates are evident before proceeding to clinical studies.

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