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# INCREASE OF SERUM BCL-2 CONCENTRATION IN SEVERE HEAD INJURY: The Role of $ACTH_{4-10}Pro^{8}Gly^{9}Pro^{10}$ and HMG Co-A Reductase Inhibitor

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**Objective:** $ACTH_{4-10}Pro^{8}Gly^{9}Pro^{10}$ and HMG Co-A reductase inhibitor had a well-known neuroprotective effects. One important process happened in head injury is apoptotic neuronal death. Bcl-2 is one of anti-apoptotic protein inhibits the intrinsic pathway of apoptosis. This study aimed to compare the effect of standard therapy, $ACTH_{4-10}Pro^{8}Gly^{9}Pro^{10}$, and HMG Co-A reductase inhibitor on serum Bcl-2 levels and the potential effect to a better outcome and reduction of hospital stay. **Method**: Subjects of severe head injury without any indication for surgery were taken consecutively (n=60) and separated into three groups of; standard treatment only (control group), standard treatment combined with $ACTH_{4-10}Pro^{8}Gly^{9}Pro^{10}$, and standard treatment combined withInhibitor HMG CoA Reductase. Blood samples were taken on day-1 and day-5 from each subject for measurement of Bcl-2 concentration. Barthel index and MMSE were measured at discharge and hospital length of stay was noted. **Results**: Bcl-2 serum levels in control group was 1.49±1.01 ng/mL on day one and 1.64±0.61 ng/mL on day five; and 1.72±1.40 ng/mL on day one and 4.02±1.19 ng/mL on day five after treatment with $ACTH_{4-10}Pro^{8}Gly^{9}Pro^{10}$. In the HMG Co-A reductase inhibitor group, Bcl-2 serum level was 1.55±0.98ng/mL on day one and 2.00±0.90ng/mL on day five. The correlation of outcome (Barthel Index and MMSE) with serum Bcl-2 levels was not significant. We found the length of stay in the $ACTH_{4-10}Pro^{8}Gly^{9}Pro^{10}$ group was significantly shorter ($p<0.05$; CI 95%). **Conclusion:** $ACTH_{4-10}Pro^{8}Gly^{9}Pro^{10}$ significantly increased serum Bcl-2 concentration in head injury. Although we didn't find any correlation between serum Bcl-2and outcome (Barthel Index and MMSE), therapy with $ACTH_{4-10}Pro^{8}Gly^{9}Pro^{10}$ resulted in a significantly shorter hospital length of stay.

**Keywords: Bcl-2, $ACTH_{4-10}Pro^{8}Gly^{9}Pro^{10}$, traumatic brain injury**

## INTRODUCTION

Traumatic brain injury (TBI) is a major public health problem in industrialized countries. Around 1.7 million people sustain TBI annually in the United States.[1] The treatment of choice and improvement of outcome in TBI remains a challenge. The clinical outcome of TBI patients itself is determined not only by the primary brain lesions, but also by the secondary brain damage.[2] Furthermore, there are evidences suggest that significant cell death may occur during a period of days to weeks after the insult due to a programmed cell death or apoptosis.[3]

Bcl-2 is a protein found in mitochondria, homologue of the c-elegans death gene-9, that inhibits the intrinsic pathway of caspase activation by stabilizing the mitochondrial membrane potential and inhibiting opening of the mitochondrial permeability transition pore.[4,5] In mammalian, Bcl-2 is the prototypic member of a family of genes with both pro (e.g., Bax, Bak and Bok) and anti-apoptotic (e.g., Bcl-xL, Bcl-w, MCL-1, and Bfl-1) properties.[4]

Expression of Bcl-2 is caused by a response to different types of injury to the CNS and neurodegenerative diseases.[6] In the controlled cortical impact model (CCI) in rodents, it is induced within hours after TBI and maintained up to 7 days in surviving neurons in cortex and hippocampal regions.[7] Bcl-2 expression is also induced in neurons that are ischemic but survive the injury.[8]

N-terminal fragments of adrenocorticotropic hormone (ACTH) – a member of the melanocortin family of peptides – are well known for their potent neuro-regenerative and cognitive activities.[9] The heptapeptide $ACTH_{4-10}Pro^{8}Gly^{9}Pro^{10}$ (Met-Glu-His-Phe-Pro-Gly-Pro) is a synthetic analogue of a short $ACTH_{4-10}$ fragment (Met-Glu-His-Phe-Arg-Trp-Gly). $ACTH_{4-10}Pro^{8}Gly^{9}Pro^{10}$ is completely devoid of any hormonal activity associated with the full-length ACTH molecule, which stimulates learning and memory formation in rodents and humans.[9-11] In addition, $ACTH_{4-10}Pro^{8}Gly^{9}Pro^{10}$ profoundly affects several forebrain and

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hippocampal functions; it increases selective attention at the moment of information reception, improves memory consolidation, and promotes learning abilities.[12]

Despite these clinical benefits, the cellular and molecular mechanisms underlying the action of $ACTH_{4-10}Pro^{8}Gly^{9}Pro^{10}$ in the brain are largely unknown. A double-blind placebo-controlled trial in 160 patients with carotid ischemic stroke (IS) confirmed the safety profile of $ACTH_{4-10}Pro^{8}Gly^{9}Pro^{10}$ in daily dose 150 mcg/kg BW for the first 5 days after the event resulting in accelerated regression of neurological symptoms, a lower 30-days-mortality, and a significantly higher proportion of patients with good recovery. Marked increase in Bcl-2, anti-inflammatory cytokines, SOD and growth factors in Cerebrospinal Fluid was also registered in the $ACTH_{4-10}Pro^{8}Gly^{9}Pro^{10}$group as well as a reduction in pro-inflammatory cytokines and CRP.[13]

3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors (statins) are the most commonly prescribed drugs used to combat hypercholesterolemia. First marketed in the USA in 1987 (lovastatin), these lipid-lowering agents are the products of *Aspergillusterreus* fermentation or synthetic production and reduce lipid levels by blocking the rate-limiting enzyme controlling cholesterol synthesis, HMG-CoA reductase. Simvastatin, introduced in the early 1990s, is one of the more potent members of the HMG-CoA reductase family of drugs and has recently been described as beneficially affecting pathologies other than hyperlipidemia. Notable work has been done to suggest that statin treatment has neurological benefits related to regeneration, improved growth, and protection from insults. Statins have been shown to improve synaptogenesis following neuronal hypoxia as a model for ischemic stroke[14] as well as increasing vascular endothelial growth factor, improving cerebral blood flow and enhancing brain plasticity.[15] Finally, chronic simvastatin treatment has been viewed as neuroprotective both in vivo[16]and in vitro.[17] Chronic in vivo administration of lipophilic (lovastatin and simvastatin) and hydrophilic (pravastatin) statins alters a number of gene expression pathways[18], and changes in these pathways may be responsible for the pleiotropic effects of statins. A particularly important and novel finding of this study was the alteration in genes regulating cell death and survival, especially Bcl-2, which was up-regulated at the mRNA level.

The goal of this study is to compare the effect $ACTH_{4-10}Pro^{8}Gly^{9}Pro^{10}$ and Simvastatin on the serum levels of Bcl-2 and the reduction of hospital length of stay. The hypothesis is that $ACTH_{4-10}Pro^{8}Gly^{9}Pro^{10}$ would increase Bcl-2 concentration and result in improved outcome with shorter hospital stay.

## MATERIALS AND METHOD

### Study design and subjects

This study was an experimental study and was approved by the Ethics Committee of the Medical Faculty, University of North Sumatera. We evaluated 60 adults with severe traumatic brain injury in our hospital. Subjects were between 18-60 years old, had a severe head injury based on Glasgow Coma Scale 3-8 with onset of accident within 48 hours before admission and had cerebral contusion as evidenced by head computed tomography, without any operative indication. Patients were excluded if they were pregnant, had history of anticoagulant use, history of neoplasm, and history of epilepsy.

Initial management was based on Advanced Trauma Life Support and every patient received standard therapy based on the prevailing consensus in the Neurosurgery Department, Medical Faculty, University of North Sumatera. Patients then were divided into three groups at random. The first group, control group, had standard therapy only. The second group, was given $ACTH_{4-10}Pro^{8}Gly^{9}Pro^{10}$ (Semax®) intranasal in addition to standard therapy for five days, at dosages of 9mg/day, 6mg/day, 3mg/day, for the remaining 3 days, respectively. The third group was given Simvastatin (Cholestat®) orally at a dosage of 40 mg each day for five days.

### Serum sample collection

Six mililiters of blood was primarily taken on the first and on the fifth day after admission for enrolled TBI subjects. Upon collection, each sample was centrifuged at 2000 rpm, 15 minutes (Eppendorf 5702), aliquoted and stored at - 20°C until the time of assay. Bcl-2 levels post-TBI were measured for a total of 60 samples.Bcl-2 was measured using immunoassay with the BCl-2 (Human) Recombinant Protein (Abnova Corporation) using Chemwell 2910 (Awareness Technology, Inc). The intra-assay coefficients of variation (CV) were <10 % for this assay. Bcl-2 serum measurement was done at the Clinical Pathology Laboratory of Adam Malik Hospital, Medan. Patients were measured by Barthel Index and MMSE score at time of discharge. Hospital length of stay was noted.

### Statistical analysis

Summary statistics, including means, standard error of the mean, and medians were computed for all continuous variables. Frequencies and percentages were determined for categorical variables. Data were checked for data errors, and normality was assessed for all continuous variables using the Kolmogorov-Smirnov (K-S) test. If distribution was normal, an Anova test was used. Otherwise, Kruskal Wallis test was applied. Correlation between continuous variables was

assessed with Pearson Correlation or Spearman, depending on the normality.

## RESULTS

The study was conducted on January 2011 until April 2012. In that period 60 patients with severe TBI were studied. The patient distribution is presented in Table 1.

Table 1
Patient distribution

| Variable | n |
|---|---|
| Age Distribution (year) | |
| 18-29 | 27 |
| 30-41 | 19 |
| 42-53 | 10 |
| 54-60 | 4 |
| Gender | |
| Male | 41 |
| Female | 19 |
| Initial GCS | 6.72 ±1.39 |

The average Bcl-2 levels on day-1 in the standard therapy group was (1.49±1.01 ng/mL), the $ACTH_{4-10}Pro^{8}Gly^{9}Pro^{10}$ treated group was (1.72± 1.40 ng/mL), and the HMG CoA reductase inhibitor group was (1.55±0.98ng/mL) are not significantly different.

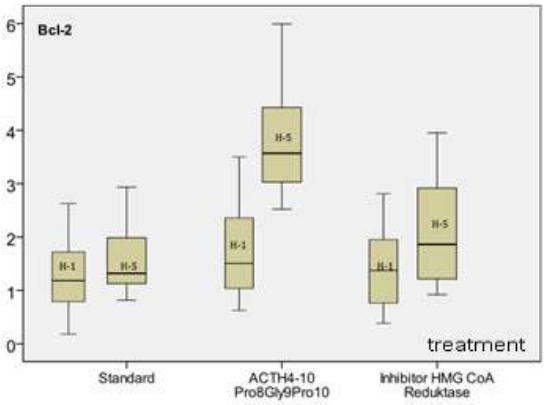


Figure 1
Bcl-2 values between treated groups on day-1 (A) and day-5 (B) in severe TBI patients

On day-5, a difference was noted between the standard therapy group (1.64±0,61ng/mL), the $ACTH_{4-10}Pro^{8}Gly^{9}Pro^{10}$ group (4,02± 1.19ng/mL), and the HMG CoA reduktase Inhibitor group (2,00± 0.90ng/mL) (Figure 1). A One Way Annova test showed that Bcl-2 levels in $ACTH_{4-10}Pro^{8}Gly^{9}Pro^{10}$group is significantly higher compared to the other two groups ($p< 0.05$; CI 95%).

Compared to day-1 an increase in Bcl-2 levels was observed on day-5 in all treated groups. No significant increase in Bcl-2 levels on day-5 was found for the standard and HMG CoA reductase Inhibitor treated group. A significant increase in Bcl-2 levels was observed in the $ACTH_{4-10}Pro^{8}Gly^{9}Pro^{10}$ ($p<0.05$). In the standard therapy group, 10 samples showed an increase in Bcl-2 levels and the remaining 6 showed a decrease. All samples in the $ACTH_{4-10}Pro^{8}Gly^{9}Pro^{10}$ group showed increased Bcl-2 levels. 17 samples in the Simvastatin group showed an increase in Bcl-2 levels and in 1 sample it is decreased (Figure 2).

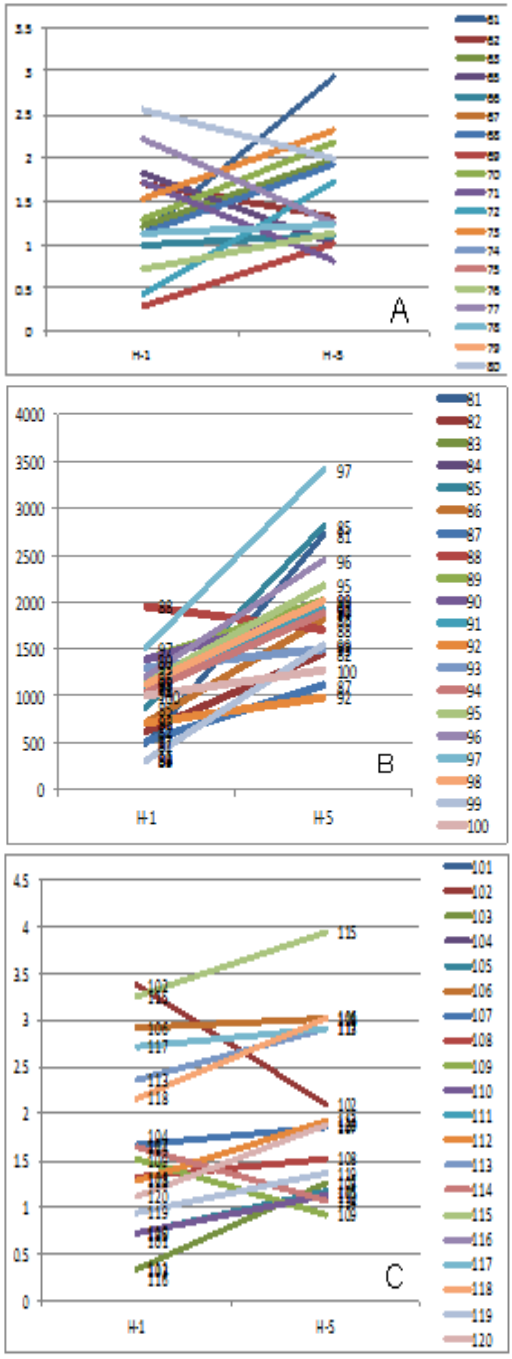


Figure 2.
Changes Bcl-2 measurements between day-1 and day-5 for the Standard therapy group (A), $ACTH_{4-10}Pro^{8}Gly^{9}Pro^{10}$ group (B), and the Simvastatin group (C)

Table 2 shows length of hospital stay in all three groups. The shortest length of stay is found in the $ACTH_{4-10}Pro^{8}$-$Gly^{9}$-$Pro^{10}$ group.

Table 2
Length of hospital stay

| Group treatment | n | Length of stay (days) | *p* |
|---|---|---|---|
| Standard | 17 | 20 ± 4.13 | |
| $ACTH_{4-10}Pro^{8}$-$Gly^{9}$ - $Pro^{10}$ | 19 | 16.94 ± 3.10 | 0.02* |
| Inhibitor HMG CoA red | 17 | 20.57 ± 3.87 | 0.600 |

## DISCUSSION

Brain injury is one of the causal factors of the high morbidity and mortality rates, particularly in young adults. To date, research is actively directed to discover optimal methods of managing brain injury, pharmacologically as well as surgically. Various neuroprotective agents have been produced to improve outcome of brain injury, such as piracetam, citicholin, pyritinol dihydrochloride monohydrate, glutamate antagonists, antioxidants, neuropeptides and caspase inhibitors. The researcher intends to study the application of a glutamate antagonist like simvastatin and a neuropeptide such as $ACTH_{4-10}Pro^{8}$-$Gly^{9}$-$Pro^{10}$by measuring the serum level of Bcl-2 in brain injury patients in relation to clinical outcome by Barthel Index , MMSE and length of hospital stay.

Our investigations show that brain injury is most prevalent in males of the 18-28 years age group, for severe as well as severe cases.In Europe, brain injury is mostly seen in males of the 15-24 years age group.[20]

### Changes in Bcl-2 levels in severe and severe cases

This study did not detect significant increases in Bcl-2 levels in severe head injury patients with standard therapy, 1.49±1.01ng/mL on day-1 and1.64±0,61ng/mL on day-5.

Uzan et al (2005) and Wagner et al (2011) conducted serial daily measurements of Bcl-2 in CSF in the first 7 days post-trauma and found that Bcl-2 levels would increase as the disease process peaked and decreased thereafter. [21,22]

In the HMG CoA reductase inhibitor group an insignificant increase in Bcl2 levels was seen from day 1 (1.55±0,98 ng/mL) and on day 5 reached (2,00±0.90ng/ml (p>0,05). Johnson-Anuna et al (2007) noted in vitro results showing HMG coA reductase having the effect protecting neurons from neural damage.This neuroprotective effect may be the result of an upregulation of Bcl2 mRNA and Bcl2 protein expression after prolonged administration. Franke et al (2006) reported a significant increase in Bcl2 after high dose (50 mg/kg BW) for 21 days in experimental animals.[23]

### Expression Pattern

In our study we observed 3 patterns of Bcl2 expression : a significant increase, slight insignificant increase and decrease. This could be because of increased expression in a group of cases, intermediate expression and decreased expression. Patterns of decrease and slight increase were mainly found in the HMG coA reductase inhibitor treated group. Increased expression was seen in the $ACTH_{4-10}Pro^{8}$-$Gly^{9}$-$Pro^{10}$ treated group.

### Group of increased Bcl2 expression

Bcl2 levels are considered increased when levels measured on day 5 show a difference of >1 ng/mL from day 1 levels. 16 subjects in the study showed increased Bcl2 expression. Increased Bcl2 expression was mostly observed in the $ACTH_{4-10}Pro^{8}$-$Gly^{9}$-$Pro^{10}$ treated group, i.e in 14samples and 2 samples in the Standard Therapy group showed significant increases

### Group of slightly increased Bcl2 expression

Bcl2 levels were considered unchanged when increases or decreases on day 5 did not exceed 1ng/ml compared to day 1. Thirty two samples remain exchanged in the severe cases, 13 in the Standard Therapy, 4 in the HMG coA reductase inhibitor group, and 15 in the $ACTH_{4-10}Pro^{8}$-$Gly^{9}$-$Pro^{10}$ group)

### Group of decreased expression of Bcl2

Bcl2 levels were considered decreased when decreases on day 5 exceed 1ng/ml compared to day 1.Two samples showed significant decrease, 1 from the Standard therapy group and 1 from the HMG coA reductase inhibitor group. No decrease in Bcl2 levels were observed in the $ACTH_{4-10}Pro^{8}$-$Gly^{9}$-$Pro^{10}$ treated group.

We didn't find any significant correlation between Bcl-2 serum level with Barthel Index and MMSE. Different form us, Clark et al (2000) observed that higher Bcl2 levels are associated with improved clinical results in children[22]. Wagner (2011) noted that significantly higher Bcl2 levels in adults are associated with improved clinical results (GOS at 6and 12 months).[20]

A shorter length of days of treatment and hospital stay was observed in the $ACTH_{4-10}Pro^{8}$-$Gly^{9}$-$Pro^{10}$ treated group compared to standard and HMG coA reductase inhibitor treated groups in both severe as well as severe cases of head injury. Gusev and Skvortsova (2003) treated acute carotid stroke patients with and also observed shorter lengths of hospital stay and reduced mortality in severe as well as severe cases.[9]

Our results in severe head injury cases appear to support those findings and empasize the importance of neuroprotective measures in managing head injury.It indicates that more studies involving a larger number of patients and

including measurements of more biomarkers to study neurodegenerative processes are needed to evaluate neuroprotective substances and their value in managing head injury.

## CONCLUSION

In conclusion, $ACTH_{4-10}Pro^{8}Gly^{9}Pro^{10}$ is increased significantly in severe head injury patients compared with standard therapy group and HMG CoA reductase inhibitor group.

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