The effect of ACTH$_{4-10}$Pro$_8$Gly$_9$Pro$_{10}$ and HMG-CoA reductase inhibitor in moderate head injury: clinical outcome and serum Bcl-2 concentration

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Abstract

Background: An important component of brain tissue damage is apoptotic neuronal death. Bcl-2 is an anti-apoptotic protein, which inhibits the intrinsic pathway of apoptosis. ACTH$_{4-10}$Pro$_8$Gly$_9$Pro$_{10}$ and HMG-CoA reductase inhibitor are known for their neuroprotective effects. This study aimed to compare the effect of standard therapy, ACTH$_{4-10}$Pro$_8$Gly$_9$Pro$_{10}$, and HMG-CoA reductase inhibitor (simvastatin 40 mg/day) on serum Bcl-2 levels, clinical outcome, and reduction of hospital stay.

Methods: Sixty subjects with moderate head injury without any indication for surgery were taken consecutively and separated into three groups: standard treatment only (control group), standard treatment combined with ACTH$_{4-10}$Pro$_8$Gly$_9$Pro$_{10}$, and standard treatment combined with inhibitor of HMG-CoA reductase. Blood samples were taken on day-1 and day-5 from each subject for measurement of Bcl-2 levels. Barthel Index and MMSE were measured at discharge and hospital length of stay was noted. Difference in mean was analyzed with one way ANOVA and correlation between Bcl-2 and clinical outcome was measured with Pearson correlation test.

Results: Bcl-2 serum levels on day-1 and day-5 were respetctively as follows: in control group were 1.39 ± 0.70 and 1.48 ± 0.77 ng/mL; in ACTH$_{4-10}$Pro$_8$Gly$_9$Pro$_{10}$ group 1.39 ± 0.70 and 3.70 ± 1.01 ng/mL; and in simvastatin group 1.53 ± 0.55 and 2.17 ± 0.56 ng/mL. Kadar Bcl-2 serum pada kelompok ACTH$_{4-10}$Pro$_8$Gly$_9$Pro$_{10}$ lebih tinggi secara bermakna dibandingkan kedua kelompok lain (p < 0.001) dan lama rawat lebih pendek secara bermakna dibandingkan tiga kelompok lainnya. Tidak ditemukan korelasi yang bermakna antara luaran klinis (indeks Barthel dan MMSE) dengan kadar Bcl-2.

Conclusion: ACTH$_{4-10}$Pro$_8$Gly$_9$Pro$_{10}$ meningkatkan kadar Bcl-2 serum secara bermakna pada cedera kepala dan peningkatan ini berkaitan dengan hari rawat yang paling pendek. Peningkatan kadar Bcl-2 serum juga terjadi pada kelompok penghambat HMG-CoA reductase (simvastatin), meskipun secara statistik tidak bermakna.

Keywords: ACTH$_{4-10}$Pro$_8$Gly$_9$Pro$_{10}$, Bcl-2, traumatic brain injury

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Traumatic brain injury (TBI) is a major public health concern in industrialized countries. It is estimated that 1.7 million people sustain TBI annually in the United States. The treatment and improvement of outcome in TBI subjects still remains a challenge. The clinical outcome of TBI patients is determined not only by the primary brain lesions, but also by the extent of secondary brain damage. Furthermore, an increasing amount of evidence suggests that significant cell death may occur during a period of days to weeks after the insult as a result of programmed cell death or apoptosis.

Bcl-2 is a mitochondrial protein, homologue of the c-elegans death gene-9, which inhibits the intrinsic apoptosis pathway and may act as a cell survival factor.

pathway of caspase activation by stabilizing the mitochondrial membrane potential and inhibiting opening of the mitochondrial permeability transition pore. In mammalian systems, Bcl-2 is the prototypic member of a family of genes with both pro (e.g., Bax, Bak and Bok) and antiapoptotic (e.g., Bcl-xL, Bcl-w, MCL-1, and Bfl-1) properties. Expression of Bcl-2 is induced in response to different types of injury to the central nervous system (CNS) and in neurodegenerative diseases. Bcl-2 protein is induced within hours after TBI in the controlled cortical impact model (CCI) in rodents and is maintained up to 7 days in surviving neurons in cortex and hippocampal regions. Bcl-2 expression is also induced in neurons that are ischemic but survive the injury.

N-terminal fragments of adrenocorticotrophic hormone (ACTH) - a member of the melanocortin family of peptides - are well known for their potent neuroregenerative and cognitive activities. The heptapeptide ACTH<sub>1-10</sub>Pro<sup>8</sup>Gly<sup>9</sup>Pro<sup>10</sup> (Met-Glu-His-Phe-Pro-Gly-Pro) is a synthetic analogue of a short ACTH<sub>4-10</sub> fragment (Met-Glu-His-Phe-Arg-Trp-Gly). ACTH<sub>4-10</sub>Pro<sup>8</sup>Gly<sup>9</sup>Pro<sup>10</sup> is completely devoid of any hormonal activity associated with the full-length ACTH molecule, which stimulates learning and memory formation in rodents and humans. In addition, ACTH<sub>4-10</sub>Pro<sup>8</sup>Gly<sup>9</sup>Pro<sup>10</sup> profoundly affects several forebrain and hippocampal functions; it increases selective attention at the moment of information reception, improves memory consolidation, and promotes learning abilities.

Despite these clinical benefits, the cellular and molecular mechanisms underlying the action of ACTH<sub>4-10</sub>Pro<sup>8</sup>Gly<sup>9</sup>Pro<sup>10</sup> 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<sub>4-10</sub>Pro<sup>8</sup>Gly<sup>9</sup>Pro<sup>10</sup> in daily dose 150 mcg/kgBW 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, superoxide dismutase (SOD), and growth factors in cerebrospinal fluid was also registered in the ACTH<sub>4-10</sub>Pro<sup>8</sup>Gly<sup>9</sup>Pro<sup>10</sup> group as well as a reduction in pro-inflammatory cytokines and C-reactive protein (CRP).

3-hydroxy-3-methylglutaryl-CoA (HMGCoA) 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 Aspergillus terreus 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 as well as increasing vascular endothelial growth factor, improving cerebral blood flow and enhancing brain plasticity. Finally, chronic simvastatin treatment has been viewed as neuroprotective. Chronic in vivo administration of lipophilic (lovastatin and simvastatin) and hydrophilic (pravastatin) statins alters a number of gene expression pathways, 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 was to compare the effect ACTH<sub>4-10</sub>Pro<sup>8</sup>Gly<sup>9</sup>Pro<sup>10</sup> and simvastatin on the serum levels of Bcl-2 and the reduction of hospital length of stay. The hypothesis is that ACTH<sub>4-10</sub>Pro<sup>8</sup>Gly<sup>9</sup>Pro<sup>10</sup> would increase Bcl-2 concentration and result in improved outcome with shorter hospital stay.

METHODS

Study design and subjects

This study was an experimental study that was approved by the Ethics Committee of the Medical Faculty, University of North Sumatera (No. 218/KOMET/FK USU/2012). We evaluated 60 adults with moderate traumatic brain injury in our hospital. Subjects were between 18-60 years old, had a moderate head injury based on Glasgow coma scale (GCS) 9-12 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(1-18)Pro8Gly9Pro10 (Semax®) intranasal in addition to 
standard therapy for five days, at dosages of 9 mg/day, 6 
mg/day, 3 mg/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 used. Correlation 
between continuous variables was assessed with Pearson 
Correlation or Spearman, depending on the normality.

RESULT

The study was conducted between January 2011 until 
April 2012. In that period 60 patients with moderate 
TBI were studied. The patient distribution can be seen 
in table 1.

The average Bcl-2 levels on day-1 in the standard therapy 
group (1.39 ± 0.75 ng/mL), the ACTH(1-18)Pro8Gly9Pro10 
treated group (1.39 ± 0.70 ng/mL), and the HMG CoA 
reductase inhibitor group (1.53 ± 0.55 ng/mL) are not 
significantly different. 

On day-5, a difference was noted between the 
standard therapy group (1.48 ± 0.77 ng/mL), the 
ACTH(1-18)Pro8Gly9Pro10 group (3.70 ± 1.01 ng/mL), 
and the HMG CoA reductase Inhibitor group (2.17 
± 0.56 ng/mL) (Figure 1). An ANOVA test showed Bcl-2 
levels to be significantly higher compared to 
the other two groups (p < 0.001).

Table 1. Patient distribution

<table>
<thead>
<tr>
<th>Variable</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age distribution (year)</td>
<td></td>
</tr>
<tr>
<td>18-29</td>
<td>29 (48)</td>
</tr>
<tr>
<td>30-41</td>
<td>17 (28)</td>
</tr>
<tr>
<td>42-53</td>
<td>7 (12)</td>
</tr>
<tr>
<td>54-60</td>
<td>7 (12)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>44 (73)</td>
</tr>
<tr>
<td>Female</td>
<td>16 (27)</td>
</tr>
<tr>
<td>Initial GCS (Glasgow coma scale)</td>
<td>10.98 ±1.43</td>
</tr>
</tbody>
</table>

![Figure 1. Bcl-2 values between treated groups on day-1 (A) and day-5 (B) in moderate TBI patients](http://mji.ui.ac.id)
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\textsubscript{4-10}Pro\textsuperscript{8}Gly\textsuperscript{9}Pro\textsuperscript{10} (p < 0.001).

In the standard therapy group, 9 samples showed an increase in Bcl-2 levels and the remaining 7 showed a decrease. All samples in the ACTH\textsubscript{4-10}Pro\textsuperscript{8}Gly\textsuperscript{9}Pro\textsuperscript{10} group showed increase of Bcl-2 levels. Fourteen samples in the simvastatin group showed increase in Bcl-2 levels and decrease in 2 samples (Figure 2).

Table 2 shows length of hospital stay in all three groups. The shortest length of stay was found in the ACTH\textsubscript{4-10}Pro\textsuperscript{8}Gly\textsuperscript{9}Pro\textsuperscript{10} group (p < 0.001).

### Table 2. Serum Bcl-2 level on day 1 and day 5

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day-1</th>
<th>Day-5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Standard</td>
<td>19</td>
<td>1.39 ± 0.75</td>
</tr>
<tr>
<td>ACTH\textsubscript{4-10}Pro\textsuperscript{8}Gly\textsuperscript{9}Pro\textsuperscript{10}</td>
<td>17</td>
<td>1.39 ± 0.70</td>
</tr>
<tr>
<td>Inhibitor HMG-CoA reductase</td>
<td>19</td>
<td>1.56 ± 0.55</td>
</tr>
</tbody>
</table>

**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, pyrtilin 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\textsubscript{4-10}Pro\textsuperscript{8}Gly\textsuperscript{9}Pro\textsuperscript{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.

![Figure 2](image_url)
Our investigations show that brain injury is most prevalent in males of the 18-28 year age group, for moderate as well as severe cases. In Europe, brain injury is mostly seen in males of the 15-24 year age group.\textsuperscript{15,16}

**Changes in Bcl-2 levels in moderate and severe cases**

This study did not find significant increases in Bcl-2 levels in moderate head injury patients with standard therapy, 1.39 ± 0.75 ng/mL on day-1 and 1.48 ± 0.77 ng/mL on day-5. Uzan, et al\textsuperscript{17} and Wagner, et al\textsuperscript{18} 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.

In ACTH\textsubscript{4-10}Pro\textsuperscript{8}-Gly\textsuperscript{9}-Pro\textsuperscript{10}group, the serum Bcl-2 levels were increased significantly, from 1.39 ± 0.70 ng/mL to 3.70 ± 1.02 ng/mL. The serum Bcl-2 levels were also increased in HMG-CoA reductase inhibitor group, but not significantly, i.e. from 1.56 ± 0.55 ng/mL to 2.17 ± 0.56 ng/mL.

Johnson-Anuna, et al\textsuperscript{19} 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 Bcl-2 mRNA and Bcl-2 protein expression after prolonged administration. Franke, et al\textsuperscript{10} reported a significant increase in Bcl-2 after high dose (50 mg/kg BW) for 21 days in experimental animals.

**Expression pattern**

In our study, we observed three patterns of Bcl-2 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\textsubscript{4-10}Pro\textsuperscript{8}-Gly\textsuperscript{9}-Pro\textsuperscript{10} treated group.

**Group of increased Bcl-2 expression**

Bcl-2 levels were considered increased when levels measured on day-5 show a difference of > 1 ng/mL from day-1 levels. Thirty nine subjects in the study showed increased Bcl-2 expression, 23 in the moderate cases group and 16 in the severe cases. Increased Bcl-2 expression was mostly observed in the ACTH\textsubscript{4-10}Pro\textsuperscript{8}-Gly\textsuperscript{9}-Pro\textsuperscript{10} treated group, i.e. in 16 samples. Five samples in the HMG-CoA reductase inhibitor treated group and 2 samples in the standard therapy group showed significant increases.

**Group of slightly increased Bcl-2 expression**

Bcl-2 levels were considered unchanged when increases or decreases on day-5 did not exceed 1 ng/mL compared to day-1. Twenty five samples remain exchanged in the moderate cases, 13 in the standard therapy, 10 in the HMG-CoA reductase inhibitor group and 2 in the ACTH\textsubscript{4-10}Pro\textsuperscript{8}-Gly\textsuperscript{9}-Pro\textsuperscript{10} group.

**Group of decreased expression of Bcl-2**

Bcl-2 levels were considered decreased when decreases on day-5 exceed 1 ng/mL compared to day 1. Four samples showed significant decrease, 2 from the standard therapy group and 2 from the HMG-CoA reductase inhibitor group. No decrease in Bcl-2 levels were observed in the ACTH\textsubscript{4-10}Pro\textsuperscript{8}-Gly\textsuperscript{9}-Pro\textsuperscript{10} treated group.

We did not find any significant correlation between Bcl-2 serum level with Barthel Index and MMSE. Different from us, Clark, et al\textsuperscript{12} observed that higher Bcl-2 levels are associated with improved clinical results in children. Wagner noted that significantly higher Bcl-2 levels in adults are associated with improved clinical results (GOS at 6 and 12 months).\textsuperscript{18}

A shorter length of days of treatment and hospital stay was observed in the ACTH\textsubscript{4-10}Pro\textsuperscript{8}-Gly\textsuperscript{9}-Pro\textsuperscript{10} treated group compared to standard and HMG-CoA reductase inhibitor treated groups in both moderate as well as severe cases of head injury. Gusev and Skvortsova treated acute carotid stroke patients with and also observed shorter lengths of hospital stay and reduced mortality in moderate as well as severe cases.\textsuperscript{9}

Our results in moderate head injury cases appear to support those findings and emphasize 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.

In conclusion, ACTH\textsubscript{4-10}Pro\textsuperscript{8}Gly\textsuperscript{9}Pro\textsuperscript{10} significantly increased serum Bcl-2 concentration in head injury. The increased of serum Bcl-2 concentration was also found in inhibitor HMG-CoA reductase group, but it was not significant. Although we did not find any correlation between serum Bcl-2 with outcome (Barthel Index and MMSE), therapy with ACTH\textsubscript{4-10}Pro\textsuperscript{8}Gly\textsuperscript{9}Pro\textsuperscript{10} resulted in a significantly shorter hospital length of stay.

http://mji.ui.ac.id
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