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The effect of ACTH$_{4-10}$Pro^{8}-Gly^{9}-Pro^{10} on spinal cord's bax concentration in sprague-dawley rat on acute spinal cord injury

Mohamad Arif Fauzi[1*], Eko Agus Subagio[1], Budi Utomo[1]

ABSTRACT

Introduction: Acute spinal cord injury (ASCI) triggers an inflammatory response that causes apoptosis. ACTH$_{4-10}$Pro^{8}-Gly^{9}-Pro^{10} has a neuromodulator effect that can inhibit apoptosis. This effect is expected to help prevent deterioration in ASCI. This study aimed to explain the effect of giving ACTH$_{4-10}$Pro^{8}-Gly^{9}-Pro^{10} to the value of Bax expression in the experimental animals with ASCI.

Methods: This study was a true experimental laboratory study with a complete randomized design factorial pattern. The subjects of the study were Sprague Dawley rats. Light and heavy compression of the spinal cord was performed on 12 subjects respectively. All subjects were then terminated and spinal cord tissue was taken for Bax analysis and histopathological cell apoptosis.

Results: In mice with mild ASCI given ACTH$_{4-10}$Pro^{8}-Gly^{9}-Pro^{10}, the results of Bax expression were 3.67 ± 2.08 and 8.67 ± 1.53 after 3 hours and 6 hours respectively, lower than the group with administration of 0.9% NaCl, which was equal to 11 ± 1 and 13.67 ± 0.58 ($p < 0.05$). In the heavy ASCI treatment group given ACTH$_{4-10}$Pro^{8}-Gly^{9}-Pro^{10} the results of Bax expression were 9 ± 2 and 12.33 ± 2.08 after 3 hours and 6 hours respectively, lower than the group with 0.9% NaCl, that was equal to 18 ± 2.64 and 19 ± 2 ($p < 0.05$).

Conclusion: Giving ACTH$_{4-10}$Pro^{8}-Gly^{9}-Pro^{10} in the first 3 hours yield greater decrease in Bax expression compared to the first 6 hours, despite insignificantly. Administration of ACTH$_{4-10}$Pro^{8}-Gly^{9}-Pro^{10} can reduce Bax expression in ASCI. Future researches are recommended.

Keywords: acute spinal cord injury, ACTH$_{4-10}$Pro^{8}-Gly^{9}-Pro^{10}, apoptosis, bax

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[1]Department of Neurosurgery, Faculty of Medicine, Universitas Airlangga, Dr. Soetomo Academic Medical Center Hospital

***Corresponding author:**
Mohamad Arif Fauzi, Department of Neurosurgery, Faculty of Medicine, Universitas Airlangga, Dr. Soetomo Academic Medical Center Hospital;
docter_ariffauzi@yahoo.com

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INTRODUCTION

Acute spinal cord injury (ASCI) is one of the causes of human morbidity that is difficult to cure and presents enormous challenges. Its clinical outcomes remain stagnant compared to other clinical advances throughout the history of modern medicine; when palliative care and functional recovery are minimal. More than one million people suffer from paralysis due to spinal cord injury in the United States. ASCI is the most common injury to people who are healthy between the ages of 19 and 29 years. Therefore, many patients survive decades with a very decreased quality of life after injury. The most common cause of spinal cord injury in the United States is activity associated with motor vehicle accidents, but more than 16% of these injuries occur due to exercise.[1]

Acute spinal cord injuries that are occured include primary injury or initial mechanical injury, followed by secondary injury, which is a series of cellular and molecular injuries that extend the severity of primary injury and result in progressive destruction of nerve tissue.[2,3] Damage to the spinal cord results in extensive proliferation of macrophages and microglia around the center of the lesion. This acute inflammatory response has at least a role in secondary spinal cord injury.[4,5] Several studies have shown evidence of widespread apoptosis in neurons, oligodendroglia and microglia. Many cells experience apoptosis several millimeters from the injured epicenter. This proves that in ASCI, the cell death process affects the pattern of damage to white matter and gray matter tissue, so understanding intracellular signaling pathways involved in the cell death process is important for developing therapeutic strategies aimed at reducing oligodendroglia tissue damage in ASCI.[6,7]

Secondary neuroprotection aimed at reducing cell death due to apoptosis includes free radical scavengers, NO-synthase blockers, local inflammatory inhibitors, statins, estrogen, erythropoietin, neurotropic factors, and neuropeptides ACTH$_{4-10}$Pro^{8}-Gly^{9}-Pro^{10}. Neuropeptide ACTH$_{4-10}$Pro^{8}-Gly^{9}-Pro^{10} is an analog synthetic molecule of ACTH (adrenocorticotropic hormone) short fragments that has no hormonal effect but has neuromodulatory effects by inhibiting apoptosis. The neuromodulatory effect of this compound is derived from an increase in the levels of Bcl-2 and BDNF, where Bcl-2 is an anti-apoptotic

regulatory protein.[8] It is expected that increased levels of Bcl-2 will decrease number of cells undergoing apoptosis, which is indicated by the lack of activated Bax as a pro apoptotic agent found in spinal cord injury. The lack of data from the literature about effect of $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} to the value of Bax expression in the experimental animals prompted us to evaluate further.

Material and Methods

This study was a true experimental laboratory study with complete randomized design factorial pattern. This research was conducted at the Laboratory of Experimental Animals, Biochemistry Department, Universitas Airlangga, Indonesia. Making paraffin blocks, cutting and preparations were carried out in the Anatomy Pathology, Dr. Soetomo Academic Medical Center Hospital. Immunohistochemical stain and analysis were conducted in the Biochemistry Departement of Brawijaya University. The subjects of the study were Sprague Dawley rats. Mild and severe compression of the spinal cord was performed on 12 subjects respectively. A total of 6 subjects from each group were then given $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10}, with details that 3 subjects received treatment in the first 3 hours and 3 other subjects in the first 6 hours. Six other subjects received similar treatment using 0.9% NaCl. All subjects were then terminated and spinal cord tissue was taken for Bax analysis and histopathological cell apoptosis.

The study was divided into three large groups, 2 treatment groups and 1 control group. On control group, experimental animal underwent spinal cord transection without any treatment given. On the first treatment group, experimental animals underwent laminectomy then were compressed using an aneurysm clip that weighs 20 grams. Compression was given for one minute. The laminectomy site was sewn back. This group was then divided into two groups. The first group was given nasal drops of NaCl 0.9% and the second group was given nasal drops of $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} at a dose of 100 µg / kg. Then each group was divided into two groups to be terminated and transection of the spinal cord was carried out at the 3rd and the 6th hour post-compression time.

On the second treatment group, laminectomy of experimental animals was then carried out by compression using an aneurysm clip that weighed 50 grams. Compression was given for one minute. The laminectomy site was sewn back. This group was then divided into two groups. The first group was given nasal drops of Aquades and the second group was given $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} nasal drops at a dose of 100 µg / kg. Then each group was divided into two groups to be terminated and the spinal cord transection was performed at the 3rd and 6th hour post-compression time.

The spinal cord was cut, fixed with formaldehyde and it was analyzed for immunohistochemical (IHC) examination for Bax. Assessment of Bax expression was carried out on the posterior horn of spinal cord with IHC examination technique, calculated per 100 cells using related monoclonal antibodies, seeing the presence of brown in the cell cytoplasm, seen with a light microscope with 1000 times magnification. The collected data was analyzed using ANOVA variance analysis test. Data normality analysis was carried out using Shapiro-Wilk test. Data on Bax expression levels were analyzed using ANOVA.

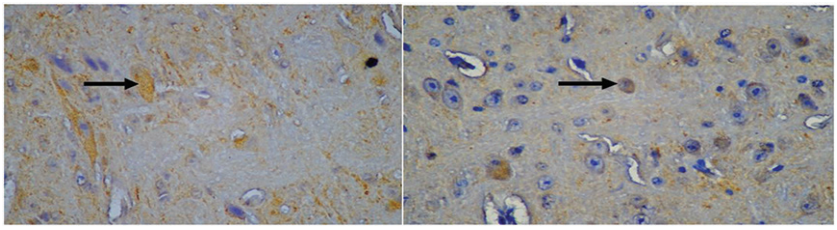

Figure 1. **A)** Results of IHC Bax staining of cross section of spinal cord of rat with treatment of mild ASCI which was given NaCl 0.9% and terminated after 3 hours. **B)** Results of IHC Bax staining of cross section of spinal cord of rat with treatment of mild ASCI which was given NaCl 0.9% and terminated after 6 hours.

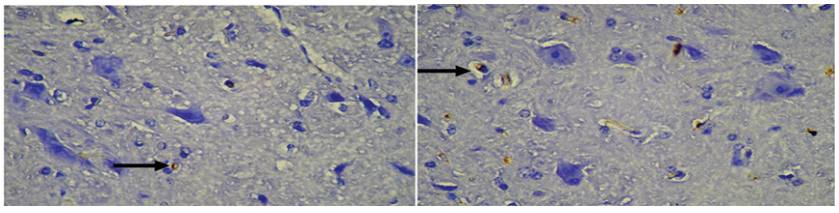

Figure 2. **A)** IHC Bax staining results of cross section of spinal cord of rat with treatment of mild ASCI which was given ACTH4-10Pro8-Gly9-Pro10 and terminated after 3 hours. **B)** IHC Bax staining results of cross section of spinal cord of rat with treatment of mild ASCI which was given ACTH4-10Pro8-Gly9-Pro10 and terminated after 6 hours.

RESULTS

Immunohistochemical stain of Bax

The following pictures are microscopic views of the spinal cord of Sprague Dawley rats with IHC Bax staining in this study. Changes that occur are designated with black arrows. Black arrows indicate neurons that positively express Bax (**Figure 1 – 4**).

Statistical test results

Figure 5 shows a graph of the assessment of Bax expression of spinal cord neuron cells in mild and

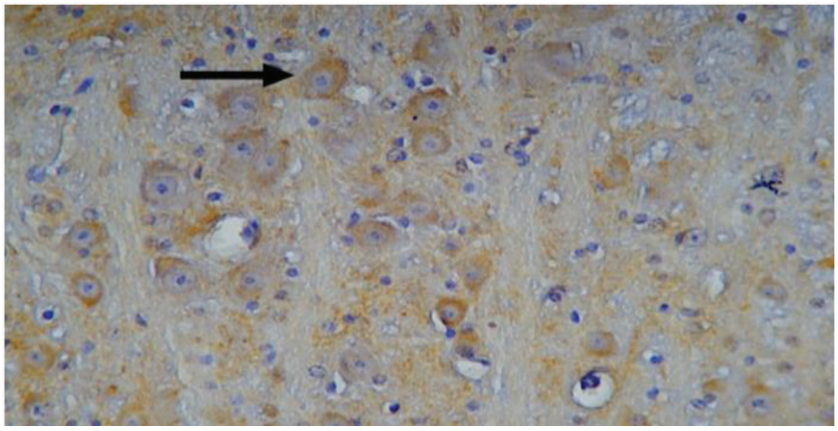

Figure 3. Results of IHC Bax staining of cross section of spinal cord of rat with treatment of severe ASCI injury which was given NaCl 0.9% and terminated after 3 hours.

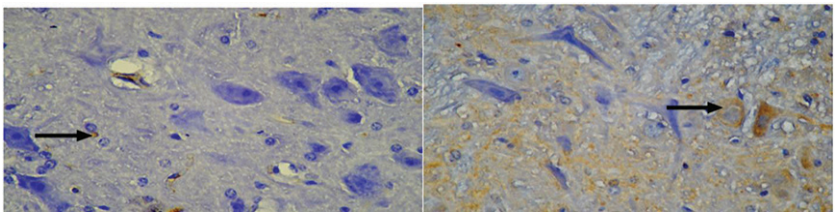

Figure 4. **A)** IHC Bax staining results of a cross section of the spinal cord of an acute injury treated with $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} and terminated after 3 hours. **B)** IHC Bax staining results of a cross section of the spinal cord of an acute injury treated with $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} and terminated after 6 hours

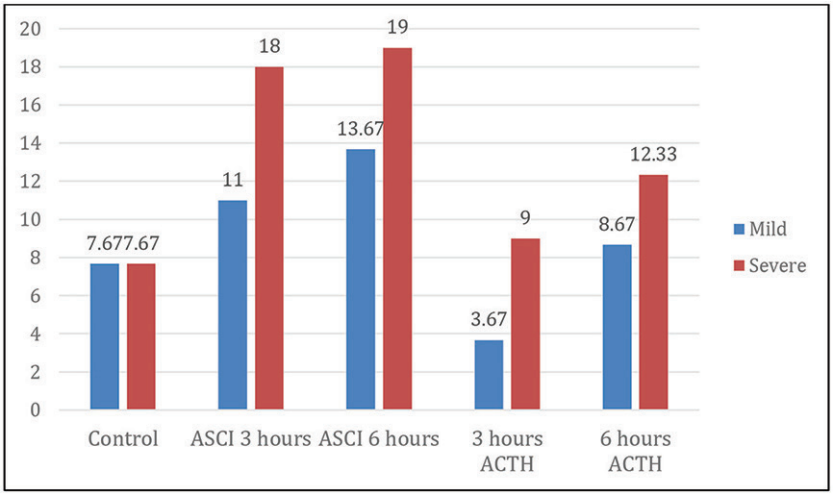

Figure 5. Graph of bax expression on mild and severe ASCI

severe ASCI divided by time of treatment and action. In the mild ASCI group which was given NaCl 0.9% and terminated after 3 hours and 6 hours, the results of Bax expression were 11 ± 1 and 13.6 ± 0.57. As for the mild ASCI group given $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} and terminated after 3 hours and 6 hours, Bax expression results were 3.67 ± 2.08 and 8.67 ± 1.53. In severe ASCI group which was given NaCl 0.9% and terminated after 3 hours and 6 hours the results of Bax expression were 18 ± 2.64 and 19 ± 2. As for the severe ASCI group given $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} and terminated after 3 hours and 6 hours, Bax expression results were 9 ± 2 and 12.33 ± 2.08.

Data distribution

The first phase of data analysis was conducted to test the normality of data distribution, using the Shapiro-Wilk test, can be seen from the following table.

Table 1. shows that all treatment groups obtained p value > 0.05 except for the mild ASCI group 0.9% NaCl 6 hours. Thus, it can be assumed the data has a normal distribution and can be used ANOVA method for data analysis, except in the group with mild ASCI NaCl 0.9% 6 hours using the Kruskal Walis method.

Table 2. shows the results of the assessment of Bax expressions for each group. Based on the results of Bax expression in each group, it can be seen that the group given $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} which was terminated 3 and 6 hours had a better mean value than the control group.

Table 3. shows the mean and SD values for mild ASCI given NaCl 0.9% after 3 hours and 6 hours. Based on the p value of the table it can be seen that the p value is significant (p = 0.027).

Table 4. shows the mean and SD values of severe ASCI given NaCl 0.9% after 3 hours and 6 hours. Based on the p value of the table it can be seen that the p value is significant (p = 0.001).

Table 5. shows the mean and SD Bax on the mild ASCI given NaCl 0.9% and $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} after 3 hours and 6 hours, along with their respective p values. From the table it can be seen that the significant p value (p < 0.05) is in both groups (after 3 hours (p = 0.032) and after 6 hours (p = 0.003).

Table 6. shows the mean and SD values of Bax in severe ASCI given NaCl 0.9% and $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} after 3 hours and 6 hours, along with their respective p values. From the table, it can be seen that the significant p value (p < 0.05) is in both groups (after 3 hours (p = 0.002) and after 6 hours (p = 0.001).

Table 7. shows the mean and SD values of the mild ASCI given $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} after 3 hours and 6 hours, along with the p value. From the table it can be seen that the differences found were not statistically significant (p = 0.069).

Table 8. shows the mean and SD Bax values on severe ASCI given $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} after 3

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Table 1. Data normality test

Treatment group	Shapiro-Wilk
Control	0.637
Mild ASCI NaCl 0.9% 3 hours	1.000
Mild ASCI NaCl 0.9% 6 hours	0.000
Mild ASCI $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} 3 hours	0.463
Mild ASCI $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} 6 hours	0.637
Severe ASCI NaCl 0.9% 3 hours	0.363
Severe ASCI NaCl 0.9% 6 hours	1.000
Severe ASCI $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} 3 hours	1.000
Severe ASCI $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} 6 hours	0.463

Table 2. Bax expression on each group

Group	n	Bax expression			
		Mean	SD	Min	Max
Control	3	7.67	1.53	6	9
Mild ASCI NaCl 0.9% 3 hours	3	11	1	10	12
Mild ASCI NaCl 0.9% 6 hours	3	13.67	0.58	13	14
Mild ASCI $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} 3 hours	3	3.67	2.08	2	6
Mild ASCI $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} 6 hours	3	8.67	1.53	7	10
Severe ASCI NaCl 0.9% 3 hours	3	18	22.64	16	21
Severe ASCI NaCl 0.9% 6 hours	3	19	2	17	21
Severe ASCI $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} 3 hours	3	9	2	7	11
Severe ASCI $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} 6 hours	3	12.33	2.08	10	14

Table 3. Comparison of bax expression on mild ASCI after 3 hours and 6 hours NaCl 0.9% treatment

Variable	Mean	SD	p
Control	7.67	1.53	
After 3 hours	11	1	0.027
After 6 hours	13.67	0.58	

Table 4. Comparison of bax expression on severe ASCI after 3 hours and 6 hours NaCl 0.9% treatment

Variable	Mean	SD	p
Control	7.67	1.53	
After 3 hours	18	2.64	0.001
After 6 hours	19	2	

Table 5. Comparison of bax expression on mild ASCI after 3 hours and 6 hours NaCl 0.9% and $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} treatment

Variable	Mild ASCI	Mean	SD	p
After 3 hours	NaCl 0.9%	11	1	0.032
	$ACTH_{4-10}Pro^{8}$–Gly^{9}–Pro^{10}	3.67	2.08	
After 6 hours	NaCl 0.9%	13.67	0.58	0.003
	$ACTH_{4-10}Pro^{8}$–Gly^{9}–Pro^{10}	8.67	1.53	

hours and 6 hours, along with the p value. From the table it can be seen that the differences found were not statistically significant (p = 0.055).

DISCUSSION

SCI treatment is a health burden which is related to complications of long immobilization, paralysis, emotional disturbances, and financial losses due to the high cost of care. Improvements in medical surgery and rehabilitation significantly help improve the quality of life of patients suffering from acute or chronic SCI, but parenchymal damage due to secondary insult to SCI has consequences for neuronal damage and long-term disability. The main goal of managements patients with ASCI is to prevent secondary injury so that it can prevent further disability. Synthetic analogs of $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} do not have hormonal activity, but can reduce Bax expression which is a potent modulator in astrocytes in synaptic plasticity.

Based on the data obtained from the results of the study, in mild ASCI mice given $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} and terminated after 3 hours and 6 hours showed lower Bax expression results than the group with NaCl 0.9%. In the mild ASCI treatment group given $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} the results of Bax expression were 3.67 ± 2.08 and 8.67 ± 1.53 after 3 hours and 6 hours terminated, lower than the group with NaCl 0.9%, that was equal to 11 ± 1 and 13.67 ± 0.58. This result was statistically significant ($p < 0.05$) with p value after 3 hours of 0.032 and p value after 6 hours of 0.003 in the comparison of Bax group expression with the treatment of $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} compared to the group with NaCl 0.9% administration.

Whereas in the severe ASCI treatment group given $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} the results of Bax expression were 9 ± 2 and 12.33 ± 2.08 after 3 hours and 6 hours terminated, lower than the group with NaCl 0.9%, which was equal to 18 ± 2.64 and 19 ± 2. This result was also statistically significant ($p < 0.05$) with p value after 3 hours of 0.002 and p value after 6 hours of 0.001. The data above showed that the mild and severe ASCI given $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} showed lower Bax expression and was statistically significant compared to the group with NaCl 0.9%.

The timing result of $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} in 3 hours and 6 hours group after being terminated in mild and severe ASCI cases was also compared. Based on the data obtained from our results, in the mild ASCI treatment group given $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} the results of Bax expression were 3.67 ± 2.08 and 8.67 ± 1.53 with p = 0.069 (no significant). In severe ASCI treatment group given $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} the results of Bax expression were

Table 6. Comparison of bax expression on severe ASCI after 3 hours and 6 hours NaCl 0.9% and $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} treatment

Variable	Severe ASCI	Mean	SD	p
After 3 hours	NaCl 0.9%	18	2.64	0.002
	$ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10}	9	2	
After 6 hours	NaCl 0.9%	19	2	0.001
	$ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10}	12.33	2.08	

Table 7. Comparison of bax expression on mild ASCI given $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} after 3 hours and 6 hours

Variable	Mean	SD	p
Control	7.67	1.53	
After 3 hours	3.67	2.08	0.069
After 6 hours	8.67	1.53	

Table 8. Comparison of bax expression on severe ASCI given $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} after 3 hours and 6 hours

Variable	Mean	SD	p
Control	7.67	1.53	
After 3 hours	9	2	0.055
After 6 hours	12.33	2.08	

9 ± 2 and 12.33 ± 2.08 after 3 hours and 6 hours terminated, with p value 0.055 (not significant). So, it can be concluded that the administration of $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} cannot reduce the Bax value if given after 3 hours compared to after 6 hours in both mild and severe ASCI cases.

In previous studies, $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} has been used to treat and prevent brain injury complications. Research on the use of $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} on ASCI is still limited, but can be one promising treatment option. The timing of $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} administration was chosen as early as the first 3 hours after SCI, this was due to consideration of glia cell culture produced from the brains of newborn mice, $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} resulted in increased BDNF at the mRNA level in 40-60 minutes so it was estimated that changes in this protein level immediately after 3 hours of administration of $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10}. This study also tried to determine the golden hours of $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} on SCI. The use of $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} is mainly to prevent secondary injuries that can interfere with nerve regeneration. Secondary injuries occur within 24 hours after the primary injury. Many factors affect secondary injuries, such as hypoxia, ischemia, oxidative stress and other inflammatory factors. The condition of this factor may differ between humans and other mammals or mice in this case.

The results of this study can be used as a reference in conducting similar research. We have not yet gotten similar study that looks at Bax's expression on the use of $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10}. This study can also be used as a comparative material to see Bax expression in other parts of the central or peripheral nerves.

CONCLUSION

Giving $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} in the first 3 hours yield greater decrease in Bax expression compared to the first 6 hours, despite insignificantly. Administration of $ACTH_{4-10}Pro^{8}$-Gly^{9}-Pro^{10} can reduce Bax expression in ASCI. Future researches are recommended.

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ETHICAL CLEARANCE

Not required for this article.

CONFLICT OF INTEREST

There is no conflict of interest related to the materials or method used in this study.

AUTHOR'S CONTRIBUTION

Authors took part in design of the study and contributed to data collection. MAF did literature review and drafted the manuscript. EAS and BU made critical revisions to the mauscript and all agree to accept equal responsibility for accuracy of the contents of this article.

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