The Effect of ACTH(4–10) PRO8-GLY9-PRO10 Administration on the Expression of IL-6 and IL-8 in Sprague Dawley Mice with Spinal Cord Injury

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Abstract

Background Spinal cord injury (SCI) is a significant cause of morbidity since it results in the inflammation process which leads to necrosis or apoptosis. Inflammatory response to the tissue damage increases IL-6 and IL-8 levels. ACTH4–10Pro8-Gly9-Pro10 is a peptide community that has been shown to have a beneficial effect on minimizing the morbidity and increasing the recovery time.

Methods This study is a true experimental laboratory research with a totally randomized method. The subjects were animal models with light and extreme compression of spinal cord, respectively.

Results The administration of *ACTH 4–10* in mild SCI in the 3-hour observation group did not show a significant difference in *IL-6* expression compared with the 6-hour observation group. The administration of *ACTH 4–10* in severe SCI showed a significantly lower expression level of *IL-6* in the 3-hour observation group compared with the 6-hour one. The administration of *ACTH 4–10* in severe SCI led to a significantly lower *IL-8* expression in the 3-hour observation group compared with the 6-hour one. However, there was no significant difference in *IL-8* expression in the group receiving *ACTH 4–10* in 3 hours observation compared with that in 6 hours observation.

Conclusion The administration of ACTH4–10Pro8-Gly9-Pro10 can reduce the expression of IL-6 and IL-8 at 3-hour and 6-hour observation after mild and severe SCI in animal models. Future research works are recommended.

Keywords
- ACTH4–10Pro8-Gly9-Pro10
- spinal cord injury
- IL-6
- IL-8

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Introduction

Spinal cord injury (SCI) carries a physical and mental burden as it causes permanent motoric and sensory dysfunctions and significantly lowers the quality of life. It causes neurological deficits through primary injury, due to irreversible, direct, and mechanical damage to the myelin during the impact, and secondary injury mechanisms.[1] From the previous study, it is known that 24 hours after trauma is the golden period to conduct a decompression surgery, and yet there has not been any strict guideline for the benefit of the surgery conducted in the acute or later phase of SCI.[2,3] There is a controversial therapy for SCI, which includes the administration of high-dose methylprednisolone that could give more side effects instead of therapeutical effects.[4] In the future, the treatment approach to SCI will involve blocking the inflammation and progressive pathogenesis of SCI, stimulating the regeneration of neurons.

Inflammation in SCI could induce the apoptosis cascade that leads to cell death. The role of inflammation in SCI's pathophysiology suggested us to test *ACTH(4–10) Pro8-Gly9-Pro10,* a neuromodulator agent that could trigger an anti-apoptosis effect. We conducted an experiment in SCI-induced *Sprague Dawley* mice and administered them with intranasal *ACTH(4–10) Pro8-Gly9-Pro10*, and later, we collected the myelin to identify *IL-6* and *IL-8* through immunohistochemistry staining.[6] This study aimed to discover more ways to treat inflammation from SCI with the possibility for its future clinical application in increasing the recovery rate and decreasing the morbidity rate.

Materials and Methods

This study is a true experimental laboratory study with a completely randomized design. It was conducted in the Animal Laboratorium Universitas Airlangga and Pathology Anatomy and Biochemistry Department in the Faculty of Medicine Universitas Brawijaya over the course of 3 months period.

The subjects were divided into the control group which did not receive any compression to the spinal cord, while sham and treatment groups received spinal cord compression on the T2 level. The control and treatment groups were divided into 20 and 35 g compression groups for mild and severe compression simulation, respectively. The sham group received a placebo using normal saline, while the treatment group received *ACTH 4–10* 300 µg/kg (P) through intranasal drops. Spinal cord transection was done for sham and treatment groups after 3 and 6 hours on the level of injury. The tissues were observed by deparaffinization and immunohistochemistry staining of IL-6 and IL-8, and the levels of *IL-6* and *IL-8* were measured under a light microscope with 400x magnification of all areas.

The data were collected in a controlled environment with the same treatment. The levels of *IL-6* dan *IL-8* were presented in a relative expression graph. The normality of the data was tested with the Shapiro–Wilk test. Normally distributed data were analyzed using the analysis of variance followed by post hoc analysis using the Tukey method.

Results

IL-6 Expression in Mild SCI

►**Table 1** shows post hoc test results of *IL-6* expression in mild SCI with the Tukey HSD method ($p < 0.05$).

Mild SCI with the administration of NaCl 0.9% in 3-hours and 6-hour observation groups and with the administration of *ACTH 4–10* in the 3-hour observation group showed a significantly higher mean of *IL-6* expression compared with controls. However, the administration of *ACTH 4–10* in mild SCI in the 3-hour observation group did not show a significant difference compared with that in the 6-hour observation group.

IL-6 Expression in Severe SCI

►**Table 2** shows post hoc test results of *IL-6* expression in severe SCI with the Tukey HSD method ($p < 0.05$).

Table 1 Post hoc test results of IL-6 expression in mild SCI with the Tukey HSD method ($p < 0.05$)

Group	Mean	Standard deviation	Group	Mean	Standard deviation	*p*-Value
Control	3.00	1.63	3 h SCI	10.43	3.05	0.001[a]
			6 h SCI	13.43	3.60	0.001[a]
			3 h SCI + *ACTH 4–10*	8.43	2.51	0.005[a]
			6 h SCI + *ACTH 4–10*	10.71	1.89	0.001[a]
3 h SCI	10.43	3.05	6 h SCI	13.43	3.60	0.235
			3 h SCI + *ACTH 4–10*	8.43	2.51	0.621
			6 h SCI + *ACTH 4–10*	10.71	1.89	1.000
6 h SCI	13.43	3.60	3 h SCI + *ACTH 4–10*	8.43	2.51	0.011[a]
			6 h SCI + *ACTH 4–10*	10.71	1.89	0.326
3 h SCI + *ACTH 4–10*	8.43	2.51	6 h SCI + *ACTH 4–10*	10.71	1.89	0.496

Abbreviation: SCI, spinal cord injury.
[a]Significant at *p*-value <0.05.

Table 2 Post hoc test results of IL-6 expression in severe SCI with the Tukey HSD method ($p < 0.05$)

Group	Mean	Standard deviation	Group	Mean	Standard deviation	*p*-Value
Control	2.86	1.07	3 h SCI	18.71	2.75	0.001[a]
			6 h SCI	27.71	1.70	0.001[a]
			3 h SCI + *ACTH 4–10*	10.71	1.38	0.001[a]
			6 h SCI + *ACTH 4–10*	16.00	2.31	0.001[a]
3 h SCI	18.71	2.75	6 h SCI	27.71	1.70	0.001[a]
			3 h SCI + *ACTH 4–10*	10.71	1.38	0.001[a]
			6 h SCI + *ACTH 4–10*	16.00	2.31	0.093
6 h SCI	27.71	1.70	3 h SCI + *ACTH 4–10*	10.71	1.38	0.001[a]
			6 h SCI + *ACTH 4–10*	16.00	2.31	0.001[a]
3 h SCI + *ACTH 4–10*	10.71	1.38	6 h SCI + *ACTH 4–10*	16.00	2.31	0.001[a]

Abbreviation: SCI, spinal cord injury.
[a]Significant at *p*-value <0.05.

IL-6 expression in severe SCI with the administration of *ACTH 4–10* in 3- and 6-hour observation groups showed a significantly lower mean compared with the administration of NaCl 0.9%. The administration of *ACTH 4–10* in severe SCI showed a significantly lower expression level of *IL-6* in the 3-hour observation group compared with the 6-hour observation group.

IL-8 Expression in Mild SCI

Tabel 3 shows post hoc test results of *IL-8* expression in mild SCI with the Tukey HSD method ($p < 0.05$).

There was a significant difference ($p < 0.05$) in all comparisons, excluding the *IL-8* expression in severe SCI with the administration of NaCl 0.9% in the 3-hour observation group compared with the administration of *ACTH 4–10* in the 6-hour observation group. *IL-8* expression in severe SCI with the administration of *ACTH 4–10* in 3- and 6-hour observation groups showed a significantly lower mean compared with the administration of NaCl 0.9% in 3- and 6-hour observation groups. The administration of *ACTH 4–10* in severe SCI led to a significantly lower *IL-8* expression in the 3-hour observation group compared with the 6-hour one.

IL-8 Expression in Severe SCI

►**Table 4** showed post hoc test results of IL-8 expression in severe SCI with the Tukey HSD method ($p < 0.05$).

In severe SCI, the administration of *ACTH 4–10* showed a significantly lower *IL-8* expression level compared with the administration of NaCl 0.9% either in 3- or 6-hour observation groups. There was no significant difference in *IL-8* expression in the group receiving *ACTH 4–10* in 3-hour observation compared with that in 6 hours.

Discussion

Spinal injury could lead to assorted injuries, including SCI. The clinical manifestation of SCI could vary from spinal

Tabel 3 Post hoc test results of *IL-8* expression in mild SCI with the Tukey HSD method ($p < 0.05$)

Group	Mean	Standard deviation	Group	Mean	Standard deviation	*p*-Value
Control	3.43	0.98	3 h SCI	12.86	1.86	0.001[a]
			6 h SCI	18.57	1.90	0.001[a]
			3 h SCI + *ACTH 4–10*	7.57	2.07	0.001[a]
			6 h SCI + *ACTH 4–10*	12.14	1.68	0.001[a]
3 h SCI	12.86	1.86	6 h SCI	18.57	1.90	0.001[a]
			3 h SCI + *ACTH 4–10*	7.57	2.07	0.001[a]
			6 h SCI + *ACTH 4–10*	12.14	1.68	0.938
6 h SCI	18.57	1.90	3 h SCI + *ACTH 4–10*	7.57	2.07	0.001[a]
			6 h SCI + *ACTH 4–10*	12.14	1.68	0.001[a]
3 h SCI + *ACTH 4–10*	7.57	2.07	6 h SCI + *ACTH 4–10*	12.14	1.68	0.001[a]

Abbreviation: SCI, spinal cord injury.
[a]Significant at *p*-value <0.05.

Table 4 Post hoc test results of *IL-8* expression in severe SCI with the Tukey HSD method ($p < 0.05$).

Group	Mean	Standard Deviation	Group	Mean	Standard Deviation	*p*-Value
Control	3.43	1.99	3 h SCI	28.71	6.42	0.001[a]
			6 h SCI	30.14	4.14	0.001[a]
			3 h SCI + *ACTH 4–10*	11.00	2.65	0.009[a]
			6 h SCI + *ACTH 4–10*	14.57	2.64	0.001[a]
3 h SCI	28.71	6.42	6 h SCI	30.14	4.14	0.958
			3 h SCI + *ACTH 4–10*	11.00	2.65	0.001[a]
			6 h SCI + *ACTH 4–10*	14.57	2.64	0.001[a]
6 h SCI	30.14	4.14	3 h SCI + *ACTH 4–10*	11.00	2.65	0.001[a]
			6 h SCI + *ACTH 4–10*	14.57	2.64	0.001[a]
3 h SCI + *ACTH 4–10*	11.00	2.65	6 h SCI + *ACTH 4–10*	14.57	2.64	0.443

Abbreviation: SCI, spinal cord injury.
[a]Significant at *p*-value <0.05.

cord commotion, contusion, laceration, hemorrhage, compression, to transection. SCI is divided into two processes involving a mechanical primary injury and secondary injury. The process of injury leads to inflammation and apoptosis.[7] Programed cell death leads to demyelination and axonal degeneration of the injury. Most of cell deaths that were studied resulted from primary injury and then continues to secondary injury. These two mechanisms are mediated by several types of inflammation, free radicals inducing cell deaths, and glutamate excitotoxicity.[5] Cytokines pathway modulates the central nervous system's inflammation by stimulating the other inflammatory cytokines, chemokines, nitric oxide, and reactive oxygen species.[8] Cytokines in leukocytes pathway that causes cell death are also the ones we studied here, which are *IL-6* and *IL-8*.

Vascular damage by SCI harms the blood–brain barrier (BBB) after which the injury's location is quickly infiltrated by neutrophils. This process of vascular damage mainly contributes to the progression of secondary injury, which the primary injury happens in the first 3–24 hours after SCI. *IL-6* and *TNF-a* are the first pro-inflammatory cytokines released in the first 30 to 45 of the injuries. Around the injured tissue, the production of *IL-6* and *TNF-a* is abundantly significant.[9] *TNF-a* modulates the apoptosis of spinal cord neurons in mice through glutamate pathway, and it was reported that the administration of *TNF* antagonists decreases the development of inflammation and tissue damage caused by SCI.[10]

The increase of *IL-1* cytokine family, like *IL-1a*, was reported as clear evidence of an important role it plays in inducing inflammation caused by SCI.[7,11,12] Central nervous system gives a response to the inflammation in SCI initiated by immune cells from peripheral tissue and activates glial cells that proliferate and migrate to the injury site after SCI.[9] T cells play an essential role in activating macrophages and modulating the cellular immune response. Macrophages and microglial cells contribute to the pathogenesis of secondary injury and cytokine release in inflammation, *TNF-α*, *IL-1*, *IL-6*, *IL-8* dan *IL-10*.[7] Thus, *TNF-a* and *IL-1* play an important role in apoptosis caused by SCI and it is mediated by a cytokine pathway.

Surgical treatment in SCI is aimed to increase the quality of life, whereas the prevention of secondary treatment is aimed to at least avoid further damage that could lead to a permanent and long-term disability that could deteriorate the patient's quality of life. The administration of *ACTH(4–10) Pro8-Gly9-Pro10* by blocking the *M2 receptor* is expected to decrease the apoptosis in SCI, so it could be considered as a therapy regimen in addition to indicated surgical treatment. The administration of *ACTH(4–10) Pro8-Gly9-Pro10* is done by intranasal drops. It works by diffusion through the olfactory tract's perineural cavity and trigeminal nerve branch, retrograde axonal transport, nose capillary microcirculation, until it reaches the central nervous system. In 1 to 4 minutes, it arrives at the BBB and cerebrospinal fluid, and the effect lasts from 20 to 24 hours even after the compound has already been eliminated.[13] It is known that secondary lesion happens in 24 hours after the primary lesion so the treatment is given as early as less than 24 hours after the primary lesion.

IL-6 Expression in Acute Spinal Cord Compression Injury

Based on the results of this study, in a mild SCI group administered with *ACTH(4–10) Pro8-Gly9-Pro10,* a statistically significant difference was observed in 3 hours administration compared with 6 hours in the expression of *IL-6*, both in mild and severe SCI. This shows that the administration time of *ACTH(4–10) Pro8-Gly9-Pro10* has a great impact on the expression of inflammatory cytokines. It can be concluded that early administration of *ACTH(4–10) Pro8-Gly9-Pro10* in both mild and severe SCI is expected to modulate inflammation leading to the decrease of secondary lesion of

neurons and glial cells from the injury. It is also concluded that in SCI, the early administration, less than 3 hours, is expected to help modulate pro-inflammatory cytokines because of the significant gap of *IL-6* expression compared with the control group.

This finding is suitable with the existing theories and previous studies that the administration of *ACTH(4–10) Pro8-Gly9-Pro10* as an anti-inflammation drug prevents secondary lesion by repairing neurons, blocking the *M2 receptor* activity, and decreasing the level of anti-inflammatory cytokines.[7] A previous study showed a drastic increase of *IL-6* expression and its receptor during an acute phase of SCI. This is linked with the function of *IL-6* that could induce the differentiation of neural stem cell into astrocyte that leads to scar formation (glial scar).[14] Another experiment conducted the suppression of *IL-6* where in vitro study shows the suppression of astrocytic differentiation, whereas in vivo study shows the inhibition of astrogliosis. The depletion of *IL-6* in SCI-induced animals also shows the decrease of inflammatory cells in the injury site and the decrease of scar formation severity and results in a better neurological repair in SCI-induced mice.[14]

IL-8 Expression in Acute Spinal Cord Compression Injury

Trauma in spinal cord leads to increase in the level of *IL-8* that functions as a chemoattractant that induces the migration of inflammatory cells like neutrophils to the inflammation site.[15] *IL-8* is produced by macrophage and somatic cell that regulate neutrophil and T cell. *IL-8* has a peak level in the first 24 hours in SCI. This is caused by the activation of microglial and neutrophils infiltrating the parenchyma cells.[7]

Based on the results of this study, in mild SCI group administered with *ACTH(4–10) Pro8-Gly9-Pro10,* a statistically significant difference was observed in 3 hours administration compared with 6 hours in the expression of *IL-8* both in mild and severe SCI, showing that the administration time of *ACTH(4–10) Pro8-Gly9-Pro10* also plays a major role in the inflammatory cytokines expression.

This finding is linked to the discovery saying that glucocorticoids play a role in blocking *IL-8*. The level of cortisol is inversely proportional to *IL-8* and other cytokines. Therefore, it is concluded that ACTH could indirectly decrease the level of *IL-8* through cortisol pathway.[16]

Conclusion

The administration of *ACTH 4–10* could decrease the expression of one or more *IL-6* and *IL-8* in SCI model animals with spinal cord compression, both in mild and severe SCI and at 3 hours and 6 hours. The decrease of *IL-6* and *IL-8* mostly in a severe compression of the spinal cord as its level increased on a higher level compared with the mild compression. *ACTH 4–10* might be a viable option to be studied further specifically for SCI in reducing glial formation and irreversible inflammatory damage. Further experiments are needed for analyzing the mechanism of the action of *ACTH 4–10* in inhibiting inflammation in SCI.

Ethical Approval
This study has not involved human subjects.

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None.

Conflict of Interest
None declared.

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