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.
**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. 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. 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. In the future, the treatment approach to SCI will involve blocking 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-inflammation 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. 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 Laboratory 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).

| Group               | Mean | Standard deviation | Group               | Mean | Standard deviation | p-Value |
|---------------------|------|--------------------|---------------------|------|--------------------|---------|
| Control             | 3.00 | 1.63               | 3 h SCI             | 10.43| 3.05               | 0.001*  |
|                     | 6 h SCI | 13.43            | 3 h SCI + ACTH 4–10 | 8.43 | 2.51               | 0.005*  |
|                     | 6 h SCI + ACTH 4–10 | 10.71            | 10.71 | 1.89               | 0.001*  |
| 3 h SCI             | 10.43| 3.05               | 6 h SCI             | 13.43| 3.60               | 0.235   |
|                     | 3 h SCI + ACTH 4–10 | 8.43             | 8.43 | 2.51               | 0.621   |
|                     | 6 h SCI + ACTH 4–10 | 10.71            | 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*  |
|                     | 6 h SCI + ACTH 4–10 | 10.71            | 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.

*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 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...
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 | p-Value |
|------------------|-------|--------------------|---------|
| Control          | 3.43  | 1.99               |         |
| 3 h SCI          | 28.71 | 6.42               | 0.001*  |
| 6 h SCI          | 30.14 | 4.14               | 0.001*  |
| 3 h SCI + ACTH 4–10 | 11.00 | 2.65               | 0.009*  |
| 6 h SCI + ACTH 4–10 | 14.57 | 2.64               | 0.001*  |
| 6 h SCI + ACTH 4–10 | 11.00 | 2.65               | 0.001*  |
| 6 h SCI + ACTH 4–10 | 14.57 | 2.64               | 0.001*  |
| 3 h SCI + ACTH 4–10 | 11.00 | 2.65               | 0.958   |
| 6 h SCI + ACTH 4–10 | 14.57 | 2.64               | 0.443   |

Abbreviation: SCI, spinal cord injury.
*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-α 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-α is abundantly significant.9 TNF-α 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-1α, 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-α 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 severe SCI.
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-inflammatory drug prevents secondary lesion by repairing neurons, blocking the M2 receptor activity, and decreasing the level of anti-inflammatory cytokines. 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). 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.

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. 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.

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.

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.

Funding

None.

Conflict of Interest

None declared.

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References

1. Okada S. The pathophysiological role of acute inflammation after spinal cord injury. Inflamm Regen 2016;36(01):20
2. Fehlings MG, Perrin RG. The timing of surgical intervention in the treatment of spinal cord injury: a systematic review of recent clinical evidence. Spine 2006;31(11, Suppl):S28–S35, discussion S36
3. Batchelor PE, Wills TE, Skeers P, et al. Meta-analysis of pre-clinical studies of early decompression in acute spinal cord injury: a battle of time and pressure. PLoS One 2013;8(08):e72659
4. Varma AK, Das A, Wallace G IV, et al. Spinal cord injury: a review of current therapy, future treatments, and basic science frontiers. Neurochem Res 2013;38(05):895–905
5. Stammers AT, Liu J, Kwon BK. Expression of inflammatory cytokines following acute spinal cord injury in a rodent model. J Neurosci Res 2012;90(04):782–790
6. Poon PC, Gupta D, Shoichet MS, Tator CH. Clip compression model is useful for thoracic spinal cord injuries: histologic and functional correlates. Spine 2007;32(25):2853–2859
7. Kwon BK, Stammers AMT, Belanger LM, et al. Cerebrospinal fluid inflammatory cytokines and biomarkers of injury severity in acute human spinal cord injury. J Neurotrauma 2010;27(04):669–682
8. Gensel JC, Zhang B. Macrophage activation and its role in repair and pathology after spinal cord injury. Brain Res 2015;1619:1–11
9. Donnelly DJ, Popovich PG. Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. Exp Neurol 2008;209(02):378–388
10. Fleming JC, Norenberg MD, Ramsay DA, et al. The cellular inflammatory response in human spinal cords after injury. Brain 2006;129(Pt 12):3249–3269
11. Ni H, Jin W, Yuan B, et al. Curcumin inhibits the increase of labile zinc and the expression of inflammatory cytokines after traumatic spinal cord injury in rats. J Surg Res 2014;187(02):646–652
12. Yang L, Jones NR, Blumbergs PC, et al. Severity-dependent expression of pro-inflammatory cytokines in traumatic spinal cord injury in the rat. J Clin Neurosci 2005;12(03):276–284
13. Ahuja CS, Wilson JR, Nori S, et al. Traumatic spinal cord injury. Nat Rev Dis Primers 2017;3(01):17018
14. Nakamura M, Okada S, Toyama Y, Okano H. Role of IL-6 in spinal cord injury in a mouse model. Clin Rev Allergy Immunol 2005;28(03):197–204

15. Kang J, Jiang MH, Min HJ, et al. IKK-β-mediated myeloid cell activation exacerbates inflammation and inhibits recovery after spinal cord injury. Eur J Immunol 2011;41(05):1266–1277

16. Schuld C, Franz S, Brüggemann K, et al; EMSCI study group. International standards for neurological classification of spinal cord injury: impact of the revised worksheet (revision 02/13) on classification performance. J Spinal Cord Med 2016;39(05):504–512