Original Article

$ACTH_{4-10}PRO^{8}$-GLY^{9}-PRO^{10} improves Neutrophil profile in spinal cord injury of rat models

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ABSTRACT

Acute spinal cord injury (SCI) is a health burden that affects daily function. Based on its prevalence, SCI becomes a major problem in the country. Inflammation is one of SCI property that stays as the main target for management in the last decade. $ACTH_{4-10}PRO^{8}$-GLY^{9}-PRO^{1} (Met-Glu-His-Phe-Pro-Gly-Pro) has been known for its anti-inflammatory properties but remains unclear for SCI models. This experimental study was done on the SCI model of the Sprague-Dawley rat. Neutrophil was taken as an inflammatory marker from the spinal cord of the rat model on 3 and 6 hours of observation following injury. Statistical analysis was done using ANOVA comparison test with significance cut off of $p < 0.05$, CI 95%.

Six samples from each control and treatment group (normal saline 3 and 6 hours; $ACTH_{4-10}PRO^{8}$-GLY^{9}-PRO^{10} 3 and 6 hours) were observed for immunoreacted values of neutrophil staining to assess inflammatory reactions in spinal cord injury models. Statistical analysis continued with the Kruskal-Wallis test and found significant differences ($p < 0.001$) between groups. Post-hoc tests were carried out using the Steel method. The most significant difference in Neutrophil levels was found in the $ACTH_{4-10}PRO^{8}$-GLY^{9}-PRO^{10} 6 hours group against normal saline 6 hours.

Keywords: Spinal cord injury, ACTH, Inflammation, Neutrophil

Introduction

The issues of prevention and complex treatment of nervous system diseases with the account of a nutritional factor are of current importance [1]. Acute Spinal Cord Injury (SCI) is a traumatic event that results in disturbance to sensory, motoric, or autonomic functions and affects the patient's social, physical, or psychological condition. SCI becomes a public health problem and a severe event for individuals because it causes permanent motor / sensory dysfunction and significantly reduces the quality of life [2, 3]. Healthy lifestyle behaviors are not only aimed at preventing a disease or illness but also at improving the overall health of a person [4, 5]. The prevalence of SCI varies 13 per 100,000 [6, 7]. The average age of patients suffering from SCI increased from 40 in 1993 to 50 years in 2012 with the proportion of elderly patients (ie, ≥65 years) also increasing [2]. At present, there are no data on SCI in Indonesia. In 2014, according to data from a local hospital in Jakarta, there were 104 cases of SCI with 37 cases of trauma and 67 cases of non-trauma [8].

SCI causes an inflammatory response that starts quickly in minutes and can extend to several months. A better knowledge of post-traumatic inflammation and how it affects secondary injuries makes the development of therapeutic strategies against

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specific targets. Quite several therapeutic modalities have been developed for acute SCI to either influence or be affected by post-traumatic inflammatory reactions. Pro-inflammatory mediators play an important role in the process and healing of SCI. Pro-inflammatory cytokines produced at the site of injury mediate the inflammatory response and can produce further tissue damage [9]. SCI could produce a sever immediate inflammation response in 6 to 24 hours [10]. Disrupted blood vessel will compromise the BSCB *(blood-spinal cord-barrier)* and quick infiltration from neutrophils followed. This could lead to the potentiation of neuronal cell death [11].

$ACTH_{4-10}PRO^{8}$-GLY^{9}-PRO^{10} (Met-Glu-His-Phe-Pro-Gly-Pro) plays a role in the hypothalamic-pituitary-adrenal (HPA) axis. Its anti-inflammatory effect is still unclear in SCI cases [9, 12]. The goal of this experiment is to investigate the effect of $ACTH_{4-10}PRO^{8}$-GLY^{9}-PRO^{10} on the level of neutrophil expression in the SCI model rats.

Materials and Methods

Procedures to the animals were in conform to the approval of the Ethical Committee of Faculty of Veterinary, Airlangga University. Thirty-two adult male Sprague-Dawley rats, weighing 260-300 g, were arbitrarily separated into the following three groups of six rats each:

- Control group (n = 6): Spinal cord was left uninjured as a baseline
- Treatment group (n = 12). The animal underwent a laminectomy at the level of 2nd thoracic vertebra, then performed extradural compression using an aneurysm clip that has been modified so that it had a clamping force of 35 g for 1 minute. The laminectomy site was closed. This group was then divided into 2 subgroups. The first group (T1) was given saline (0,9 NaCl) intranasally, the second group (T2) was given $ACTH_{4-10}PRO^{8}$-GLY^{9}-PRO^{10} (Semax®) at a dose of 300 µg / kg. Then in each group, T1 and T2 have divided again into two groups to be terminated and spinal cord transection was carried out at the 3 h and the 6 h after compression respectively.

Paraffin block sample collection

Spinal cord tissue was washed with phosphate-buffered saline (PBS) 3-5 times to clean from contaminants, then fixed on 10% formalin. Then dehydration was done using ethanol level (30%, 50%, 70%, 80%, 96% and absolute concentration) 60 minutes respectively. The clearing was done using xylol 2 times for 60 minutes each. Then infiltration was continued by using soft paraffin for 60 minutes at 48oC. A block in hard paraffin was made and was allowed to stand for a day. The next day it was cut 4-6 µm thickness by using a rotary microtome. The object-glass was mounted with 5% gelatine. The glass of the object produced by the paraffin block was immersed in xylol 2 times each for 5 minutes. After that, rehydration was done using serial alcohol (absolute, 96%, 80%, 70%, 50% and 30% concentration) for 5 minutes respectively. Then rinse in dH_2O for 5 minutes.

Neutrophil expression

The number of cells expressing neutrophils carried out by immunohistochemical examination techniques is counted per 100 cells using related monoclonal antibodies viewed with a 400 times magnification light microscope.

Statistical analysis

Data analysis was performed on SPSS Statistics software version 24 for Mac (IBM Corp., Armonk, NY, USA). The test of normality was performed using the Kolmogorov-Smirnov test. The expression of neutrophil concentration data was analyzed using ANOVA if the distribution was normal. Otherwise, the Kruskal-Wallis test was used. The results of data collection are presented in the form of mean ± standard deviation (SD). Significant correlation of p-value <0.05 with 95% CI.

Results and Discussion

Statistical analysis

Six samples from each control and treatment group (normal saline 3 and 6 hours; $ACTH_{4-10}PRO^{8}$-GLY^{9}-PRO^{10} 3 and 6 hours) were observed for immunoreacted values of neutrophil staining to assess inflammatory reactions in spinal cord injury models **(Figure 1)**.

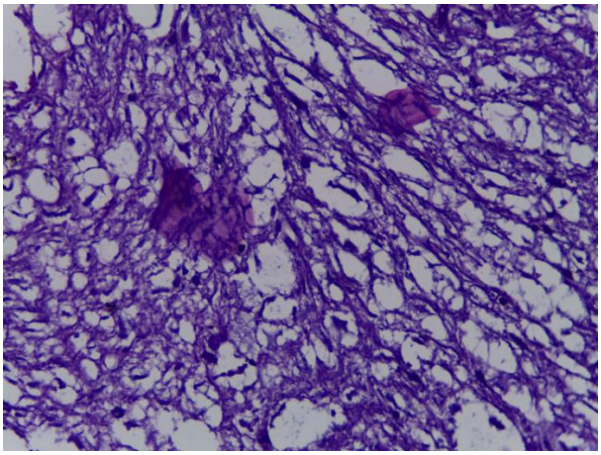

Figure 1. Positively stained neutrophil by hematoxylin-eosin

A normality test was carried out on the results obtained to see the distribution of data. The results of the normality test with the Shapiro-Wilk method showed abnormal data distribution. Obtained a median value for the control of 0.05 (0 - 0.2); $ACTH_{4-10}PRO^{8}$-GLY^{9}-PRO^{10} 3 hours amounted to 0.25 (0 - 0.4); $ACTH_{4-10}PRO^{8}$-GLY^{9}-PRO^{10} 6 hours is 0.2 (0.1 - 0.3); Normal saline 3 hours in the amount of 0.1 (0 - 0.1); and normal saline 6 hours in the amount of 0.05 (0 - 0.1). Statistical analysis continued with the Kruskal-Wallis test and found significant differences (p <0.001) between groups **(Table 1)**. Post-hoc tests were carried out using the Steel method. The

most significant difference in Neutrophil levels was found in the $ACTH_{4-10}PRO^{8}$-GLY^{9}-PRO^{10} 6 hours group against normal saline 6 hours **(Table 2)**.

Table 1. ANOVA analysis of neutrophils in the spinal cord injury model in Sprague-Dawley rats observed within 3 and 6 hours.

Group	Number of Neutrophils (IRS/FV)			p
	Median	Min	Max	
Kontrol	0.05	0.00	0.20	0.001
ACTH4-10Pro^{8}-Gly^{9}-Pro^{10} 3 hours	0.10	0.00	0.10	
ACTH4-10Pro^{8}-Gly^{9}-Pro^{10} 6 hours	0.05	0.00	0.10	
Normal saline 3 hours	0.25	0.00	0.40	
Normal saline 6 hours	0.20	0.10	0.30	

IRS/LP: *Immunoreactive score*/Field of View

Table 2. Comparison test of neutrophils between groups by the Mann-Whitney method.

Group	Mean Rank	Group	Mean Rank	p
Kontrol	4.67	ACTH4-10 3 hours	3.50	0.248
	4.00	ACTH4-10 6 hours	3.00	0.317
	2.33	Normal saline 3 hours	5.80	0.044
	3.83	Normal saline 6 hours	5.58	0.322
Normal saline 3 hours	5.80	Kontrol	2.33	0.044
	7.00	ACTH4-10 3 hours	2.50	0.010
	6.00	ACTH4-10 6 hours	2.00	0.020
	7.80	Normal saline 6 hours	4.50	0.081
Normal saline 6 hours	5.58	Kontrol	3.83	0.322
	6.83	ACTH4-10 3 hours	3.50	0.051
	6.00	ACTH4-10 6 hours	3.00	0.083
	4.50	Normal saline 3 hours	7.80	0.081
ACTH4-10 3 hours	3.50	Kontrol	4.67	0.248
	4.00	ACTH4-10 6 hours	4.00	1.000
	2.50	Normal saline 3 hours	7.00	0.010
	3.50	Normal saline 6 hours	6.83	0.051
ACTH4-10 6 hours	3.00	Kontrol	4.00	0.317
	4.00	ACTH4-10 3 hours	4.00	1.000
	2.00	Normal saline 3 hours	6.00	0.020
	3.00	Normal saline 6 hours	6.00	0.083

SCI is damage to the spinal cord due to trauma both directly and indirectly that causes dysfunction, such as motoric, sensory, autonomic, reflex functions [10, 13]. The mechanism of the occurrence of SCI is generally divided into primary and secondary mechanisms. The primary mechanism is generally affected by the impact force due to persistent compression which can develop into secondary damage. Secondary damage is an advanced process of primary injury, such as neurogenic shock, vascular abnormalities, ischemia, electrolyte disorders, and others [13].

Inflammation of the spinal cord injury causes the release of inflammatory cytokines from leukocytes that are localized to the area of injury. These pro-inflammatory cytokines are produced by cells as an initial response to injury. Cytokines released will cause an increase in the inflammatory cascade through cellular responses from T, B cells, macrophages, and other effector cells [14, 15]. The inflammatory process that arises in expressed inflammatory cytokines will induce further immunogenic responses in the injured spinal cord [16]. In the central nervous system, microglial cells that have a phagocytic function also play a role in the pathophysiological process of secondary injury and inflammation. The cytokines expressed also contribute to the release of reactive oxygen species (ROS) which act as free radicals capable of causing neuronal death [17]. SCI can cause a strong inflammatory response with the appearance of peripheral immune cells such as neutrophils (6-24 hours). The torn blood vessels damage the BBB (blood-brain-barrier) and the location of the damage is quickly infiltrated by neutrophils. This process contributes to secondary damage that occurs after primary damage. 30-45 minutes after SCI, inflammatory cells can begin to express inflammatory cytokines that can potentiate nerve cell death with the development of inflammation and the incidence of tissue injury associated with SCI [16, 18].

When an SCI occurs, especially during the initial injury, mechanical damage to the skin, muscles, and spinal cord, the

neutrophil expression will begin with a few hours and reach a peak at 24 hours. Activation of inflammatory cells, in general, is a function as hemostasis of the body, which removes damaged tissue, facilitates neutrophil cells and regulates the healing response of fibroblasts (in the case of central nervous system injuries in the form of astrocytes), platelets, and endothelial cells to the release of pro-inflammatory cytokines [19].

Very few papers focus on the role of neutrophils in the SCI model, but their adverse actions are mainly highlighted as effects/consequences of treatments and other conditions. Indeed, in most conditions, lower neutrophil accumulation in the lesion is associated with reduced pro-inflammatory cytokines, reduced apoptosis and oxidative stress, and significant motor recovery. The role of the NF-κB signaling pathway is related in this regard, both in neutrophil invasion and neutrophil activity in the lesion. Indeed, the NF-κB kinase β (IKKβ) subunit blockade neutralizes chemokine ligand 1 (CXCL1) and subsequent neutrophil infiltration, but also proinflammatory gene expression, while increasing tissue preservation and motor function [20].

The neutrophil count in our study showed a significantly lower level within 6 hours in the ACTH4-10Pro8-Gly9-Pro10 group **(Figure 2)**. Research conducted by Fleming *et al.* showed an increase in the activity of perivascular neutrophil injuries 0 - 4 hours after the incident in the area close to the injury. In that study also seen the expression of matrix metalloproteinases from neutrophils in the acute phase of injury. Neutrophils diffuse diffusely at the site of [15]. Our study shows the potential for the administration of ACTH to suppress inflammatory reactions with lower neutrophil levels in the acute phase, especially the 6-hour observation.

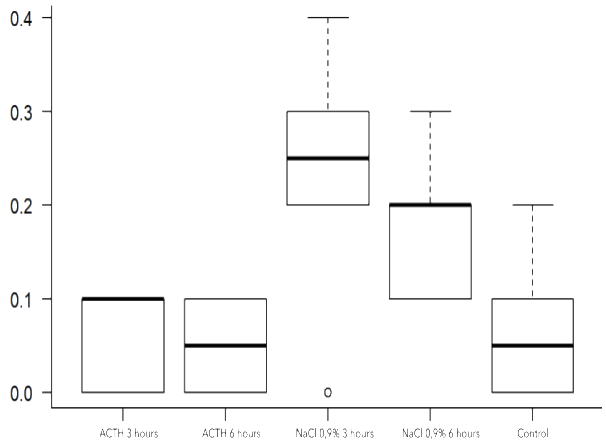

Figure 2. Boxplot of neutrophil on rat SCI model during 3 and 6 hours observation

Another finding of concern is the result of neutrophils which seem low on the results of the IHC staining from our study. These findings are consistent with findings from a study by Machado *et al.* that found relatively low neutrophils in spinal cord tissue compared with blood levels in animals after ACTH injection. From the results of the Machado analysis, it is seen that the $ANXA1^{high}$ neutrophil has a weaker chemotaxis function compared to the number of $CXCR4^{high}$ neutrophils that have a normal function in circulation. Neutrophils d $ANXA1^{high}$ act as anti-inflammatory neutrophils that do not migrate into injured tissue [21].

Secondary spinal cord damage caused by neutrophils and macrophages in animal studies is partly caused by oxidative and proteolytic enzymes. Myeloperoxidase (MPO), a well-known oxidative enzyme, is abundantly expressed by neutrophils and other phagocytes and produces hydrochloric acid which kills pathogens, but also damages surrounding tissue. Another damaging oxidative enzyme produced by inflammatory cells is nicotinamide adenine dinucleotide phosphate (NADPH) [22].

Conclusion

$ACTH_{4-10}PRO^{8}$-GLY^{9}-PRO^{10} improves neutrophil profile in spinal cord injury of rat models in 6 hours after treatment.

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Conflict of interest: None

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Ethics statement: All procedures were in accordance with the ethical standards on human experimentation in the Helsinki Declaration of 1964 and its later derivat. Informed consent was obtained from all patients included in the study.

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