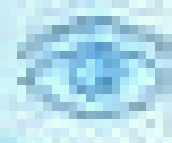


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Sanglah General Hospital

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## Animal model of contusion compression spinal cord injury by Yasargil aneurysm clip



I Nyoman Semita<sup>1,2</sup>, Dwikora Novembri Utomo<sup>3\*</sup>, Heri Suroto<sup>3</sup>, Parama Gandi<sup>4</sup>

### ABSTRACT

**Background:** Animal spinal cord injury (SCI) models have shown to be invaluable in better understanding the mechanisms related to traumatic SCI and evaluating the effectiveness of experimental therapeutic interventions. The use of clip compression can produce contusion–compression SCI models in rats with clinical features in the form of total paralysis, retention of micturition, and retention of defecation. This study aimed to validate the effects of the duration of Yasargil aneurysm clip application on the formation of SCI models with analyzed neuropathic pain, locomotor function, histology, and tumor necrosis factor (TNF- $\alpha$ ).

**Methods:** We did true experimental study investigated 20 Sprague Dawley divided into normal, 30-second, 60-second, and 90-second groups. Contusion–compression model of SCI post-laminectomy was done in 0, 30, 60, and 90 seconds using a Yasargil aneurysm clip, with a force of 65g (150kDyne). Data were analyzed using SPSS version 25 for Windows.

**Result:** We found that the locomotor expression did not indicate total paralysis after compression durations of 0 and 30 seconds, while compression durations of 60 and 90 seconds could result in total paralysis. There was no significant difference in the mean BBB scores between the compression durations of 60 and 90 seconds ( $p=1.000$ ). In addition, there was no significant difference in the mean RGS value between the 60-second model group and the 90-second model group on days 21 and 28 ( $p=1.000$ ;  $p=0.900$ ). The histological pictures at compression durations of 60 and 90 seconds show severe damage to spinal cord continuity. There was no significant difference in the mean value of TNF- $\alpha$  between the duration compressions of 60 and 90 seconds ( $p=0.937$ ).

**Conclusion:** The use of Yasargil aneurysm clips for 60 and 90 seconds could produce a contusion–compression SCI model with expressions of neuropathic pain, locomotor function, histology, and pro-inflammatory cytokine.

**Keywords:** Spinal Cord Injury Model, Contusion–Compression, Neuropathic Pain, Locomotors, TNF- $\alpha$ .

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<sup>1</sup>Doctoral Program of Medical Science, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia;

<sup>2</sup>Department of Orthopaedic and Traumatology, Faculty of Medicine, Universitas Jember, Jember, Indonesia;

<sup>3</sup>Department of Orthopaedic and Traumatology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia;

<sup>4</sup>Department of Cardiology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia;

\*Corresponding author:

Dwikora Novembri Utomo;  
Department of Orthopaedic and Traumatology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia;

[dwikora-novembri-u@fk.unair.ac.id](mailto:dwikora-novembri-u@fk.unair.ac.id)

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### INTRODUCTION

Many types of animal spinal cord compression methods use clips, balloons, spinal cord strapping, and calibrated forceps that produce spinal cord injury (SCI) similar to the persistent common human SCI.<sup>1</sup> Cheriyan T et al. stated that there are five types of SCI models: contusion, compression, dislocation, transection, and chemical. The contusion–compression model includes clip, calibrated forceps, and balloon compression.<sup>2</sup>

SCI can result in permanent neurologic deficits; complete SCI neurological recovery is still less than 1% and 90% permanent disability.<sup>3</sup> There is no definite treatment for SCI. Various research studies are ongoing, including pathophysiology,

mechanism of neuroregeneration, neuroprotective and neuro-regenerative agent, test the safety and efficacy of potential therapies.<sup>4</sup> Therefore, to be insightful for a given human condition, a disease or injury model should be similar to the human analog in terms of causation and function and must have advantages over simple clinical observation.<sup>5</sup> To select an appropriate model and test a specific hypothesis, all existing SCI animal models and related outcome assessments must be considered.<sup>6</sup>

Previous studies of SCI animal models have shown that contusion SCI was the most common model of injury (41%), compression (19.4%) and transection (32.5%). The SCI animal models were rats (88.4%), rabbits (2.4%), dogs (2.3%), and pigs (1.5%). Behavior outcome

assessments are locomotor test (89.2%), sensory test (16.3%), sensory-motor test (13.9%), autonomic test (6.7 %), and reflex-response based test (5.6%). The injury levels were thoracic (81%) and cervical (12%).<sup>6</sup> Contusion devices are designed to produce transient and acute injury to the spinal cord. In contrast, compression models are prolonged cord compression, an acute impact followed by persisting cord compression. Contusion–compression model is seen in human SCI caused by fracture-dislocations and burst fractures.<sup>2</sup>

The use of a modified aneurysm clip to generate SCI was first described in rats in 1978. The procedure was started by performing a laminectomy at the desired spine level. Then the clip is closed at a specific force around the spinal cord,

producing an acute injury.<sup>2,7</sup> Clips with a wide range of closing forces are available, generating varying closure forces and injury severities in the animal. The 50g clip compression forces produce severe injuries, while 35g has moderate injuries.<sup>2</sup> Abdullahi D et al. stated that aneurysm clip compression could result in contusion-compression SCI models.<sup>1</sup> Oliveri RS et al. observe that because there are similarities between rodents and humans in terms of electrophysiological, functional, and morphological results, mice can be CMS experimental animals.<sup>8</sup> However, there is no certainty about the duration of compression to create the SCI model. Thus, this research aimed to analyze the effects of the duration of Yasargil aneurysm clip application in SCI models with neuropathic pain, locomotor, histology, and biomarker TNF- $\alpha$  expressions.

## METHODS

The research was a true experimental study. The sample size was counted by the Lemeshow formula ( $n=5$  rats), with correction factors of 20%. The sampling technique uses random allocation with simple random, namely the numbering method (lottery). The rats were randomly grouped into the following four groups: normal group (five experimental rats did not have SCI); 30-second group (five experimental rats were given an SCI model for 30 seconds); 60-second group (five experimental rats were given an SCI model for 60 seconds); and 90-second group (five experimental rats have given an SCI model for 90 seconds). All groups were replicated five times. We observed the study over 28 days. The study's independent variable is the duration of compression, whereas the dependent variables are neuropathic pain, locomotor function, histology, and TNF- $\alpha$  cytokine. The study protocol was reviewed and approved by the university (REC.1112/UN25.8/KEPK/DL/2021). All rats were approved by the animal health office (No.503/A.1/0005. B/35.09.325/2020).

### Rats and SCI Models

The adult male *Rattus norvegicus* pure strain Sprague Dawley rats were three to four months old and weighed 300–350g. The inclusion criteria were male, 3–4

months old, 300–350 grams weight, pure line, and healthy with a health certificate from a veterinary polyclinic doctor. And the exclusion criteria were experimental rats that had received immunomodulatory therapy and were fatally ill. Acclimatization was carried out for seven days by one laboratory technician and two veterinarians. Rats were kept in separate cages, one cage containing one rat, using a plastic box, 45x30x15cm<sup>3</sup>, with woven wire as a cover. The floor mat was covered with wood shavings and an underpad to absorb urine and maintain moisture. The room comfort was provided by air conditioning to maintain the room temperature of 22°C  $\pm$  2°C and a humidity of 50–70%. An exhaust fan was used to remove the smell of ammonia. The environment is a quiet room with a 12-hour cycle of light and dark. The light sources are 300 lux electric lamps 1 meter from the floor. The cages were cleaned every three days with soap and running water. Feed was 30–35g of pellets (10% of BW) and 30–35 ml of mineral water (10% of BW).

Contusion-compression of the spinal cord was conducted using the commercially available spinal cord impactor Yasargil aneurysm clip, with a length of 7mm and a load of 65g (equivalent to 150kDyne). The animals were anesthetized with ketamine (75mg/kg) and acepromazine (ACP) (3mg/kg) intraperitoneal. The rats were placed on a fixation board in a prone position, and the back hair was shaved to approximately 3cm. The operating area was disinfected with 10% betadine and 75% alcohol. The surgical level was marked by tracing the level of the T12 rib to the T12 spinous process using a 2cm skin incision. A level of T10–T11 partial laminectomy was conducted to expose the level of the T10 spinal cord. The tip of the titanium Yasargil aneurysm clip was placed at a distance of 1mm from the spinal cord anteriorly and posteriorly. The spinal cord was impacted suddenly for 30, 60, and 90 seconds by tip retraction using an applicator. This action produced an SCI contusion-compression model with the dura appearing cloudy white and impacted flat. The operating field was cleaned using saline, and the muscle and skin were sutured together in layers.

### Neuropathic Pain Assessment

The Rat Grimace Scale (RGS) was used to observe the neuropathic pain. It uses animal facial expressions consisting of four measures: orbital/eye lifting, nose and cheek protrusion, ear position, and whisker position. It is based on the presence of each action unit, denoted by a score of 0, 1, or 2. The score is “0” if there is no change (normal), “1” if the change is moderate, and “2” if the change is clear. The score was measured before surgery and on days 21 and 28 after surgery. Each rat was observed using a video camera for nine minutes in each testing session. The assessment was carried out every 15 seconds, followed by a 15-second interval. Every three minutes, the score was averaged, and then the total score was averaged as the final score.

### Locomotor Assessment

To assess the locomotor expression, the BBB open-field test was performed on days 1, 7, 14, 21, and 28 after injury. The BBB measures the tail, body, legs, trunk stability, limb movement, and toe clearance, all of which are examined to measure locomotor abilities. The score shows a range of numbers between 0 and 21. A score of 0 is no movement, and a score of 21 is normal movement without a locomotor disorder.

### Hematoxylin-eosin (HE) Staining Procedure

The histopathological operational procedure consists of three phases: tissue processor, network incision, and hematoxylin-eosin (HE) staining. The detailed procedures are explained below.

1. Tissue process or procedure
  - Turn on the appliance by pressing the power button to the on position.
  - Raise the white needle up until the tool cap is in the top position/not moving.
  - Insert the cassette that contains the tissue in the metal basket.
  - Make sure the basket containing the specimen is completely submerged in the liquid in the glass container.
  - Set the desired basket position by pressing the red needle down.



- Press down on the white needle until the cap is completely down.
  - Set the program to start position at 15.00 WIB and finish at 6.00 WIB.
2. Network incision procedure
- Prepare paraffin blocks to be cut on a cold plate.
  - Prepare a tissue rotation bath containing water at a temperature of 43°C.
  - Prepare the hot plate at a temperature of 60°C
  - Prepare the object glass according to the label of the specimen being cut.
  - Adjust the thickness of the incision on the rotary microtome.
  - Adjust the blade angle on the rotary microtome.
  - Attach the disposable blade to the microtome blade holder.
  - Place the paraffin block on the microtome paraffin block holder.
  - Perform trimming on sliced paraffin blocks.
  - Perform paraffin box incisions several times.
  - Take it with the incision obtained.
  - Place the incision on the tissue flotation bath.
  - Take 1-2 well-inflated incisions with the appropriate labeled object glass.
  - Place the object glass containing the tissue incision on the hot plate for 15 minutes.
  - Histo PA preparations are ready for staining.
3. Hematoxylin-eosin (HE) staining procedure
- Deparaffinize with xylol.
  - Hydrate with alcohol to 70%–96%.
  - Immerse in running water for 10 minutes.
  - Put in Meyer's Hematoxylin for 15 minutes.
  - Wash under running water.
  - View with an ordinary microscope.
  - Put in 1% eosin solution for 30 seconds.
  - Dehydrate with alcohol 80%–96%.
  - Clear with xylol.
  - Mount with EZ-Mount medium.

### Enzyme-linked Immunosorbent Assay (ELISA) examination

The specimen for ELISA was collected from cardiac blood. The TNF- $\alpha$  analysis used the serum. The ELISA kit for the TNF- $\alpha$  analysis used the sandwich ELISA protocol. TNF- $\alpha$  was evaluated using quantitative measurements. The Standard International (SI) used to evaluate the TNF- $\alpha$  is  $\mu\text{g/ml}$ .

### Statistical methods

The data in this research is reported as the mean  $\pm$  standard deviation of the mean. "Statistical Package for Social Science (SPSS) software (Version 25, IBM)" was utilized to analyze the differences between groups. A p-value of  $< 0.05$  was considered statistically significant.

### RESULT

Neuropathic pain measured by RGS at days 21 and 28 were used to determine the effects of the compression duration with Yasargil aneurysm clips on the SCI model. The statistical test showed no significant difference between the 60-second and 90-second groups ( $d_{21}=p>1.000$ ,  $d_{28}=p>0.900$ ). The normal and 30-second groups had a significance of  $p = 0.008$ , the normal and 60-second group had a significance of  $p=0.000$ , the normal and 90-second group had a significance of  $p=0.000$ , and the 30-second and 60-second groups had a significance of  $p=0.000$  as seen in [Figure 1](#).

The rats were examined over four weeks to assess the recovery of their motor function. Locomotor recovery started on day 7 and continued until day 28. The mean BBB score of the normal group was  $x: 21$ ; of the 30-second group, it was  $x: 17.8$ ; and of the 60-second group was  $x: 1.2$ , whereas the mean score of the 90-second group was  $x: 1.2$  as seen in [Figure 2](#).

Based on the pairwise comparison table, there is no significant difference ( $\alpha=0.05$ ) in the mean value of the BBB locomotor score between the 90-second model group and the 60-second model group ( $p=1.000$ ;  $p>0.05$ ). Meanwhile, there was a significant difference between the normal model group and the 30-second model group ( $p=0.000$ ), between the normal model group and the 60-second model group ( $p=0.000$ ), between the

normal model group and the 90-second model group ( $p=0.000$ ), between the 30-second model group and the 60-second model group ( $p=0.000$ ), and between the 30-second model group and the 90-second model group ( $p=0.000$ ) as shown in [Table 1](#).

The histological pictures at compression durations of 60 and 90 seconds show severe damage to spinal cord continuity, whereas the compression duration in the 30-second group resulted in partial damage to spinal cord continuity, as seen in [Figure 3](#).

Based on the mean value, the level of TNF- $\alpha$  in the normal group  $\bar{x} = 105.069$   $\mu\text{g/ml}$ , 30-second model group  $\bar{x} = 172.048$   $\mu\text{g/ml}$ , 60-second model group  $\bar{x} = 214,705$   $\mu\text{g/ml}$ , and 90-second model group  $\bar{x} = 220,705$   $\mu\text{g/ml}$ . Based on the multiple comparison table, there was no significant difference in the mean value of TNF- $\alpha$  examination between the 60-second model group and the 90-second model group ( $p = 0.937$ ;  $p > 0.05$ ). Meanwhile, there was a significant difference between the normal model group and the 30-second model group ( $p = 0.001$ ), between the normal model group and the 60-second model group ( $p = 0.000$ ), between the normal model group and the 90-second model group ( $p = 0.000$ ), between the 30-second model group and the 60-second model group ( $p = 0.035$ ), and between the 30-second model group and the 90-second model group ( $p = 0.015$ ) as shown in [Table 2](#).

### DISCUSSION

The study of the Yasargil aneurysm clip found that the locomotor expression did not indicate total paralysis after compression durations of 0 and 30 seconds, while compression durations of 60 and 90 seconds could result in total paralysis. There was no significant difference in locomotor expression between the compression durations of 60 and 90 seconds ( $p = 1.000$ ;  $p > 0.05$ ). There was no significant difference in the mean RGS value between the 60-second model group and the 90-second model group on days 21 and 28 ( $p = 1.000$  and  $p = 0.900$ ). The histological pictures at compression durations of 60 and 90 seconds show severe damage to spinal cord continuity.

There was no significant difference between the compression durations of 60 and 90 seconds in pro-inflammatory cytokine TNF- $\alpha$ , with a significance value of  $p = 0.937$ .

Contusion devices are designed to

produce transient and acute injury to the spinal cord. In contrast, compression models are prolonged cord compression, an acute impact followed by persisting cord compression.<sup>2</sup> In all the different methods of compression models.<sup>9</sup> Anjum

A et al. stated that the SCI pathophysiology includes ischemia, inflammation, apoptosis, and glial scar formation.<sup>10</sup> This process begins with trauma that results in microvascular damage in the form of bleeding, thrombosis, and vasospasm.<sup>11</sup> This microvascular damage causes the spinal cord to undergo hypoperfusion, hypoxia, and ischemia. Ischemia in the spinal cord affects cellular and molecular inflammation processes, neuron and neuroglia cell apoptosis, and glial scar formation, which mechanically and chemically inhibits SCI regeneration.<sup>10,11</sup>

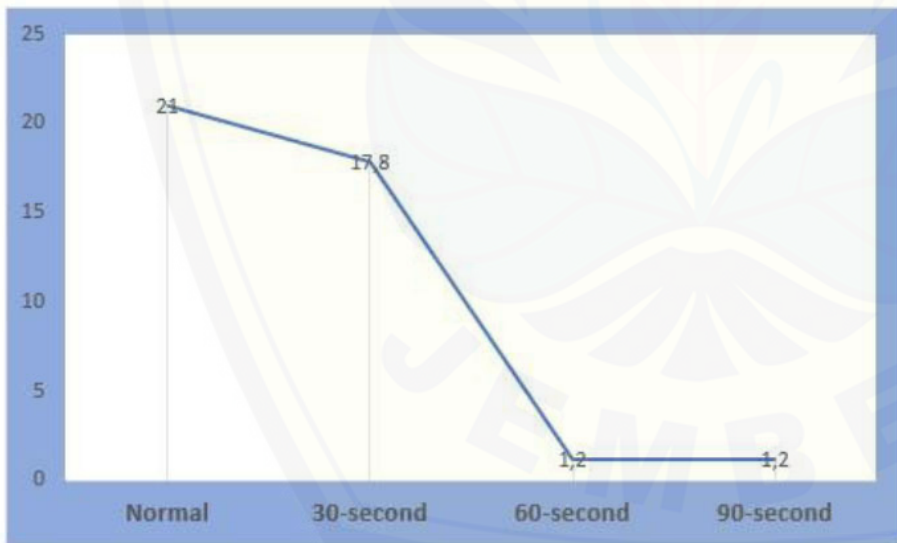
The use of a modified aneurysm clip to generate SCI was first described in rats in 1978. The procedure was started by performing a laminectomy at the desired level of the spine; then, the clip is closed at a specific force around the spinal cord, producing an acute injury.<sup>2,7</sup> Clips with a wide range of closing forces are available, generating varying closure forces and injury severities in the animal. Forces of 50g clip compression produce severe injuries, while 35g produce moderate injuries.<sup>2</sup> The advantages of clip compression are that it is relatively inexpensive, generates SCI of varying severity and is adaptable at all levels.<sup>2</sup> A clip compression technique can also be used to occlude the blood supply to inflict ischemia model SCI.<sup>2,6</sup>

This model research shows severe damage in the spinal cord continuity histological pictures at compression durations of 60 and 90 seconds. Jazayeri SB et al. stated that histopathologic evaluation revealed less tissue loss and more preserved tissue in the 3-second group compared to the 10-minute group.<sup>12</sup> This is reinforced by the finding of Ahmed RU et al. that the compression duration of 60 seconds gives a histological picture of moderate to severe discontinuity.<sup>7</sup>

Locomotor function using BBB evaluation has been used by many researchers in the world because it is more effective than using the incline plane test or the flexor withdrawal reflex.<sup>13,14</sup> This study showed significant neuropathic pain outcomes after using the Yasargil aneurysm clip on the spinal cord at compression durations of 60 and 90 seconds, compared to durations of 0 and 30 seconds. Borsook D et al. stated that the RGS has high validity across different models, assessors,



**Figure 1.** Mean Rat Grimace Scale (RGS) values.



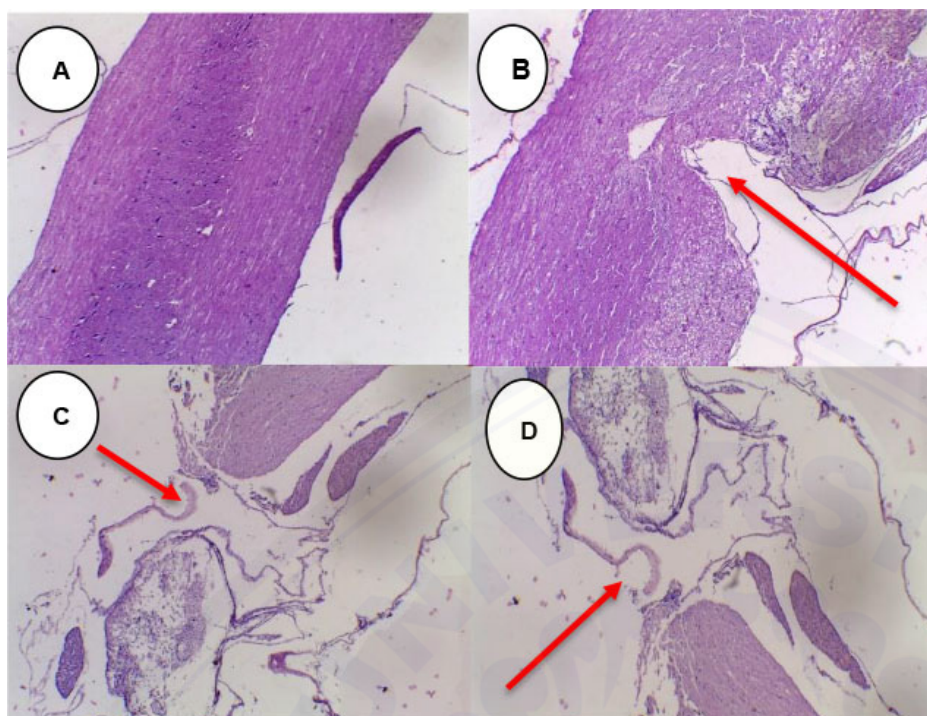
**Figure 2.** Mean BBB score values.

**Table 1.** Pairwise comparison test.

Group	Mean Difference	Std. Error	p
Normal – 30 seconds	3.20	0.557	0.000*
Normal – 60 seconds	19.8	0.557	0.000*
Normal – 90 seconds	19.8	0.557	0.000*
30 seconds – 60 seconds	16.6	0.557	0.000*
30 seconds – 90 seconds	16.6	0.557	0.000*
90 seconds – 60 seconds	2.84	0.557	1.000

Values are presented as mean difference (MD) and p-value; \* $p < 0.05$ . significant differences between groups by Pairwise Comparison Test.





**Figure 3.** (A) Histopathology normal model group (0 seconds). (B) Histopathology 30-second model group. (C) Histopathology 60-second model group. (D) Histopathology 90-second model group.

**Table 2. Tukey HSD test results of TNF- $\alpha$ .**

(I) Intervention	(J) Intervention	Mean Difference (I-J)	Std. Error	p
Normal	30 second	-66.979000	14.014335	0.001*
	60 second	-109.636800	14.014335	0.000*
	90 second	-115.636800	14.014335	0.000*
30 second	Normal	66.979000	14.014335	0.001*
	60 second	-42.657800	14.014335	0.035*
	90 second	-48.657800	14.014335	0.015*
60 second	Normal	109.636800	14.014335	0.000*
	30 second	42.657800	14.014335	0.035*
	90 second	-6.000000	14.014335	0.973
90 second	Normal	115.636800	14.014335	0.000*
	30 second	48.657800	14.014335	0.015*
	60 second	6.000000	14.014335	0.973

Values are presented as mean difference (MD) and p-value; \*p < 0.05, significant differences between groups by Tukey HSD test.

and environments.<sup>15</sup> Pain intensity can be assessed using the visual analog or numeric rating scales.<sup>16</sup> In experimental animal models of spinal cord injury, clinical pain assessment can be done using the RGS. A previous study evaluated pain using a mouse grimace scale in spinal cord injury with the T10 contusion method after cell cycle inhibition treatment.<sup>17</sup> The RGS score accurately distinguishes between experimental animals with pain

and those without pain.<sup>18</sup>

SCI causes a pathophysiological process in the form of a primary injury stage that causes damage to the gray and white matter, then develops into a secondary injury stage starting with the release of proinflammatory cytokines and recruitment of immune cells to the site of the injury.<sup>19</sup> Hellenbrand DJ et al. stated that the secondary injury stage is characterized by an inflammatory

process that triggers apoptosis of neurons, astrocytes, oligodendrocytes, and the formation of glial scars.<sup>20</sup> The most influential proinflammatory mediator in SCI is TNF- $\alpha$ , followed by other proinflammatory mediators such as IFN- $\gamma$ , IL-6, and IL-8.<sup>21</sup> Increased levels of TNF- $\alpha$  in the secondary injury stage also contribute to the induction of apoptosis.<sup>22</sup>

During spinal cord injury, TNF- $\alpha$  is secreted excessively, especially by M1 macrophages/microglia, which can induce neuroinflammation. Increased levels of the proinflammatory cytokine TNF- $\alpha$  release will affect the progression of secondary injury to SCI, thus worsening inflammation.<sup>23</sup> TNF- $\alpha$  can also induce cytotoxic events that will cause apoptosis in neurons, astrocytes, and oligodendrocytes. This can increase the formation of glial scars in the spinal cord, affecting the delivery of impulses and causing a decrease or loss of motor, sensory, or autonomic function.<sup>24-26</sup>

Generally, using a 7mm long Yasargil aneurysm clip with a load of 65g (150 kDyne) and a duration of 60 and 90 seconds optimally produces a contusion-compression SCI rat model. This is in accordance with previous research conducted by Borhani-Haghighi M et al. and Asadi-Golshan R et al., using 60 seconds and effectively providing an SCI model.<sup>13,27</sup> The limitation of this study is its small sample size. The evaluation of rat model outcomes in this study not only evaluated locomotor function but also sensory, reflex, and biological expression. The results of this study can be used in future animal studies to analyze the pathophysiology, neuroregeneration, and treatment of SCI.

## CONCLUSION

The use of Yasargil aneurysm clips with a force of 65g (150kDyne) and a duration of 60 and 90 seconds could produce a contusion-compression SCI model with expressions of neuropathic pain, locomotor function, histology, and pro-inflammatory cytokines.

## CONFLICT OF INTEREST

The author reports no conflicts of interest in this work.

## ETHICAL CONSIDERATION

This study was approved by the Ethics Committee of the Biomedical Veterinary Laboratory, Faculty of Dentistry, University of Jember (REC.1112/UN25.8/KEPK/DL/2021). All rats were approved by the animal health office (No.503/A.1/0005.B/35.09.325/2020).

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## AUTHOR CONTRIBUTION

INS contribution in the concepts, design, definition of intellectual content, literature search, experimental studies, data acquisition, data analysis, statistical analysis, manuscript preparation, and manuscript editing; DNU contribution in the concept, design, definition of intellectual content, manuscript review, a guarantor; and HS contribution in the concept, design, definition of intellectual content, manuscript review.

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