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The relationship of tumor necrosis factor alpha levels and neutrophils with skin wound age caused by sharp trauma



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ABSTRACT

Background: Sharp force injuries are the second most common cause of trauma after blunt force injury. TNF- α is a cytokine that triggers the expression of adhesion molecules, other cytokines and chemokines by endothelial cells, stopped and migrated to damaged tissues or infections that trigger inflammation. Furthermore, the initial population of leukocytes leading to the wound comprises mostly neutrophils. This study aimed to assess the correlation between TNF- α levels in the blood and skin tissues and neutrophils number with the wound age of wounds caused by sharp trauma.

Methods: This study is an experimental study using animal models. The samples were divided into the control group and the group in which the blood and the skin tissue samples were taken 1-, 3-, 12-, 24-, 48- and 72 hours after being sharp traumatized. The Elisa method examined the samples from the blood, and the skin of the right back of the mice was examined for TNF- α levels. Others from the left-back skin of the mice were microscopically examined to assess the number of neutrophils.

Results: The TNF- α level from the skin samples were detected in wound tissue as the peak levels were in the 24 hours post-traumatic group. The TNF- α levels from blood samples indicated a significant difference in TNF- α levels in each group ($p < 0.05$). However, TNF- α levels from skin samples showed no significant difference between each group. From the microscopic examination, there was a significant difference in the infiltration of neutrophil cells in all groups ($p < 0.05$). The neutrophils peak at 24 hours post-traumatic.

Conclusion: There was no significant correlation between the TNF- α levels and the number of neutrophils with the wound age in wounds caused by sharp traumas.

Keywords: ELISA, Neutrophils, Tumor Necrosis Factor- α , Wound age.

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INTRODUCTION

Sharp force injuries are the second most common cause of trauma after blunt force injury. They are caused by sharp objects or instruments that can result in incised, stab and chopping wounds. All types of injuries that arise depend on the tool used and the method of abuse.¹ When viewed from the medicolegal aspect of examining wounds caused by sharp objects, a doctor must explain various important points such as the type of wound, the type of violence, a description of the shape of the weapon that caused the wound and the classification of the injuries.²

Determination of wound's intra-vitality and age is important in the field

of forensic medicine. The wound healing were started in the first few minutes after the injury and could belast for several days until even years.³ The skin can repair its damaged tissue through wound healing process. This event consist of many steps like coagulation, inflammation, proliferation and remodeling phases. In this mechanisms, there are several cytokines and growth factors are release, including Interleukin-1 β (IL-1 β), Tumor Necrosis Factor (TNF), and other Growth Factors.⁴

Proinflammatory cytokines has an important role in inflammation process. These proteins can be applied in the intra-vitality and wound age

determination. Cytokines are mediators that have several functions, including initiating and influencing many biological events such as inflammation and wound healing.⁵ They are produced by various cell types in skin, such as inflammation cells, epithelial, endothelial and connective tissue cells. Tumor Necrosis Factor- α (TNF- α) and other cytokines play an important role in the wound healing process. Cytokines are markers that can be increased, especially in the early stages of wound healing. Cytokines like TNF- α can be used as a markers of the wound healing.³ TNF- α is a cytokine that triggers the expression of adhesion molecules, other cytokines and chemokines by

endothelial cells so that leukocyte cells can be stopped and migrate to damaged tissues or infections that trigger inflammation.

Cellular infiltration is also considered one of the important components in the estimation of wound age.⁶ In the inflammatory process, inflammatory cells like neutrophils, monocytes and lymphocytes are attracted. After hemostasis occurs, local vasodilatation will reveal due to the coagulation process and the components of blood clotting proteins. Bradykinin and complement components C3a and C5a increase vascular permeability and recruited neutrophils and monocytes to the injury area. Local vascular endothelial cells then break down their cell junction, increasing capillary permeability and inflammatory cells' margination to the wound area. The early population of leukocytes leading to the wound is composed mostly of neutrophils.⁷

This study aimed to evaluate the relationship between TNF- α levels in blood and skin tissue and the number of neutrophils with wound age in sharp force injuries. Hopefully, this research can provide information and a scientific basis on the role of TNF- α and neutrophils in the development of wounds caused by sharp trauma, especially in determining the age of wounds, which is an important issue in the forensic field.

MATERIALS AND METHODS

Study design

This study design was an experimental research with a posttest-only control group design. Totally, 28 mice that used in this study were divided into seven groups. Each group consisted of 4 mice i.e. control group, 1 hour, 3 hours, 12 hours, 24 hours, 48 hours and 72 hours after being traumatized. This study followed the Helsinki Declaration guideline and was approved by the Ethical Committee of Health Research Medical Faculty of Hasanuddin University with registered number: 233/UN 4.6.5.31/PP 36/2021.

Animal model

Male albino strain mice (*Mus musculus*) aged 4-8 weeks and weighing 32-35 grams were included in this study. The exclusion criteria were animals with stress, illness

and death before this study began. Mice were kept in open, humid and well-ventilated cages with 12 hours life / light cycle. Food and drink were given ad libitum.

Experimental procedure

The mice in this study were traumatized to make sharp injuries using punch biopsy 5 mm in the right and left-back. Blood serum samples were taken from the orbital vein. Skin samples around punch injury in the right and left-back were taken by cutting into 1cm square area. Both blood and skin samples were taken after 1 hour, 3 hours, 12 hours, 24 hours, 48 hours and 72 hours in traumatized groups and immediately in the control group. Blood serum and right back skin samples were collected to measure TNF- α levels by ELISA method using a TNF- α detection kit (BT Lab). Skin samples from left-back mice were fixated with 10% neutral buffer formalin and processed into paraffin specimens with Hematoxylin-Eosin (HE) staining. HE staining slides from skin specimens were used to measure neutrophil numbers microscopically.

Data analysis

Data were collected and analyzed statistically using the ANOVA parametric test to compare TNF- α levels and the Kruskal Wallis and Mann-Whitney U test to compare neutrophil numbers in each group. A p-value < 0.05 was considered statistically significant.

RESULTS

In this study, it was found that the lowest levels of TNF- α from blood samples were found in the 3-hour post-trauma group with a mean of 63.09 compared to TNF- α from blood samples in the control group with an average of 192.72. The 72 hours post-trauma group was the group with the highest TNF levels of all groups, with a mean of 234.11 (Table 1).

Meanwhile, skin samples found the lowest TNF levels in the 3 hours post-trauma group, with an average of 53.75. The highest TNF level from skin samples in the treatment group was found in the 24 hours post-trauma group, with a mean of 263.62. TNF levels then decreased in the group 48 hours post-trauma to the group

72 hours post-trauma with an average of 65.62 (Table 2).

On microscopic examination of skin samples from experimental animals, it was found that there was an infiltration of inflammatory cells, especially neutrophils, around the wound area (Figure 1).

This study revealed that no neutrophils were found in the skin samples from the control group of mice. The lowest neutrophil number was found in the 1-hour post-trauma group with a mean of 5 neutrophils. The highest number of neutrophils was found in the 24-hours post-trauma group, with an average of 712.33 neutrophils. The number of neutrophils then decreased in the 48 hours post-trauma group (mean 74.66) and the 72-hour post-trauma group (mean 18.33) (Table 3).

Based on Spearman's correlation analysis, it was found that there was no relationship between TNF- levels in blood serum and skin tissue and the number of neutrophils on wound age in wounds caused by sharp trauma.

DISCUSSION

Based on a statistical analysis of TNF- α levels in both blood and skin samples, the TNF- α levels in the control group were higher than in all post-traumatic groups. It shows that even though all experimental animals are already under quarantine surveillance and declared healthy during the observation, their body condition cannot be controlled. Therefore, it is necessary to carry out other examinations to ensure whether the experimental animals are healthy and that there are no diseases that affect TNF- α levels.

Severe malnutrition, cancer and metabolic diseases, consumption of glucocorticoids and cytostatic agents, and chronic radiation exposure can inhibit the wound healing process by affecting the release time and amount of cytokines.³ Many factors can affect the wound healing process, including age, history of pre-existing diseases and previous treatment or drug consumption. Technically, sampling and laboratory procedures can also affect the analysis of the test results.⁸

In our study, the maximum levels of TNF- from skin samples were in the 24-hour post-traumatic wound group.

Previous studies have found that TNF- α is produced in wound tissue, reaches peak levels at 24 hours, and then returns to basal levels.⁴ In this study, it was also seen that TNF- α levels from skin samples were low in the 72 hours post-traumatic group.

After trauma, cytokines are synthesized and released in areas of tissue damage. Several processes follow this event at the systemic level, such as the inflammatory process. Therefore, measuring the cytokine response directly on skin wounds is advisable. Although there are several references to cytokines in general, only a few studies using this method. The main problem that is often found in the availability of efficient extraction. Cytokines concentrations that obtained are minimal and relatively unstable, so

it could be difficult to obtain amounts above the ELISA detection limit. However, the skin is a fairly resistant tissue. Thus, cytokine analysis of the wound site can be very useful, at least when the skin is still looking fresh until about one-week postmortem.⁵

There are various reasons for a rapid local increase in pro-inflammatory factors. Some cells such as multipotent keratinocytes, mast cells, sweat gland epithelial cells, and some macrophages contain large amounts of various cytokines. Various mediators are stored in these cells in the inactive precursors or active form.⁵

Cytokines are signaling molecules that are important for the wound healing process. More than 30 cytokines involved in this process are produced

by macrophages, platelets, fibroblasts, epidermal cells, and neutrophils.⁹ Among the proinflammatory cytokines, TNF- α is a cytokine that is rapidly produced by vascular endothelial cells, keratinocytes and fibroblasts in the wound area, and stimulates the inflammatory phase by recruiting leukocytes into the injured tissue.⁴

TNF- α levels from blood samples in the post-traumatic group showed fluctuating results in our study. TNF- α was high in the 12 hours post-traumatic group, low at 24 hours post-trauma and finally high again in the 48-hour and 72-hour post-trauma groups. These fluctuating results may be due to factors that influence cytokines at a systemic level, such as genetics, biological age and psychological stress. Cytokine

Table 1. TNF- α levels in blood serum samples.

Groups	TNF- α levels (pg/ml)			p*
	Minimum	Maximum	Mean	
Control	111.22	351.98	192.72	0,043
1 hour Post Sharp Trauma	53.43	149.92	99.17	
3 hours Post Sharp Trauma	40.57	101.46	63.09	
12 hours Post Sharp Trauma	86.71	198.04	128.51	
24 hours Post Sharp Trauma	0	111.61	74.42	
48 hours Post Sharp Trauma	109.53	287.58	181.08	
72 hours Post Sharp Trauma	112.90	416.10	234.11	

*ANOVA test, Significant $p < 0.05$.

Table 2. TNF- α levels in skin samples.

Groups	TNF- α levels (pg/ml)			p*
	Minimum	Maximum	Mean	
Control	41,70	818,45	302,52	0,135
1 hour Post Sharp Trauma	40,58	71,80	56,94	
3 hours Post Sharp Trauma	37,37	91,56	53,75	
12 hours Post Sharp Trauma	38,71	243,29	112,24	
24 hours Post Sharp Trauma	68,87	390,57	263,62	
48 hours Post Sharp Trauma	110,78	300,88	174,18	
72 hours Post Sharp Trauma	51,61	76,85	65,62	

*ANOVA test, Significant $p < 0.05$.

Table 3. Neutrophil / PMN number in skin samples.

Groups	Neutrophil number			p*
	Minimum	Maximum	Mean	
Control	0	0	0 ^{1,2,3,4,5}	0,021
1 hour Post Sharp Trauma	0	15	5	
3 hours Post Sharp Trauma	5	385	132,33	
12 hours Post Sharp Trauma	16	223	113	
24 hours Post Sharp Trauma	479	876	712,33	
48 hours Post Sharp Trauma	4	202	74,66	
72 hours Post Sharp Trauma	12	22	18,33	

* Kruskal- Wallis test, Significant $p < 0.05$.

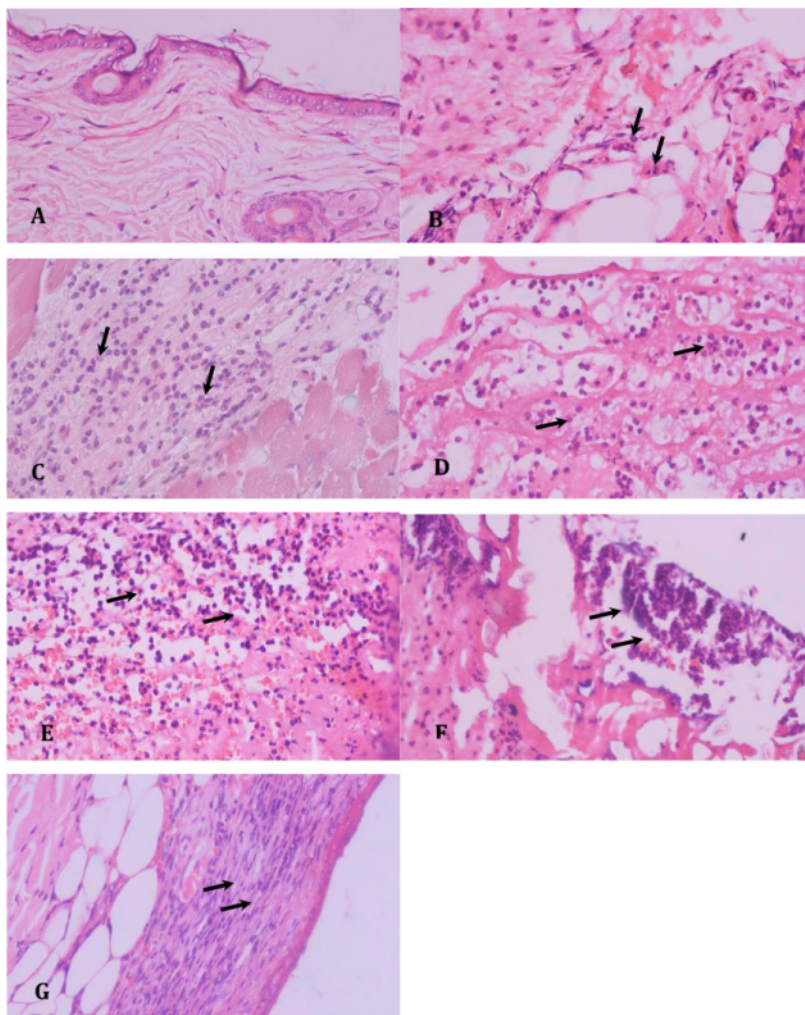


Figure 1. Infiltration of Neutrophil (PMN) in the skin wound area, microscopically. (A) Control, (B) 1 hour -, (C) 3 hours -, (D) 12 hours -, (E) 24 hours -, (F) 48 hours -, and (G) 72 hours - post sharp trauma (HE Staining, 10x objectives).

activation and production could be reduced by older age and psychological stress.⁵ By looking at these results, analysis of pro-inflammatory cytokines in injury tissue can contribute in determining wound age and vitality, especially²³ in the early phase of trauma and later phases of the wound healing process.

Although the results from skin samples did not show any significance in this study, TNF- α levels from skin samples were more in line with the expected results compared to using blood samples. TNF- α levels from blood samples reflect systemic conditions

that several factors and conditions can influence in other organs rather than local skin samples. A previous study looking for a relationship between changes in cytokine levels and wound age during wound healing in rat skin, reported that levels of IL-2 and TNF- α increased at 30 minutes after trauma, and peaked at 3 hours for IL-2 and 1 hour. For TNF- α after trauma, rebound levels were demonstrated at 48 hours.¹⁰

Many studies with various techniques for determining wound age have been published, with some contradictory

results. Because the wound healing process is complex and involves various cellular, physiological and biochemical processes, it is necessary to conduct research with different methods and use a larger number of samples and better grouping of wounds.

Cellular infiltration has been considered one of the important components for estimating wound age.⁶ Bradykinin, and the anaphylatoxins C3a and C5a increase vascular permeability and attract neutrophils and monocytes to the wound area. Local endothelial cells degrade their cell junction and increase the permeability and margination of inflammatory cells to the wound site. Neutrophils are a type of leukocyte cell that is found most early in the wound area.⁷

The presence of inflammatory cells around the wound area is the only standard histologic finding derived from an antemortem process.⁸ A previous study showed that neutrophils and macrophages could be detected in the early phase of wound healing ranging from 30 minutes to 3 hours after trauma.⁶

In our study, it was seen that neutrophil infiltration began to appear around the wound in the skin tissue in the 1-hour post-trauma group and it increased in the 3-hour post-trauma group. Neutrophil infiltration was found more and more in the skin wound tissues of the 24-hour post-traumatic group. The number of neutrophils decreased in injury tissues in the 48-hour post-traumatic group and decreased again in the 72-hour post-traumatic group.

The inflammatory phase of the wound healing process is characterized by the migration of inflammatory cells into the damage area. Neutrophils are leukocytes that first appear in large numbers in the first 24 hours.⁹ The inflammatory cells will release more proinflammatory cytokines such as TNF- α and Interleukins in the wound area. Under normal circumstances, neutrophils will disappear from the wound area after three days through the process of apoptosis, and the inflammatory phase begins to decrease.¹¹

Our study also showed no neutrophil infiltration in the skin tissue of the non-trauma control group. The same result was found in another study which showed no neutrophils were found in the tissue

observed in a few minutes or less than 1 hour after injury.⁸

The main function of neutrophils is to clean wounds by phagocytosis the potentially infectious agents and removing the remnants of damaged tissue. Within 24-48 hours, monocytes will leave vascular and differentiate into macrophages to migrate into the wound. These cells act as key regulators of the wound healing response and perform various important functions such as: removing debris and apoptotic cells including used neutrophils, helping to fight infection agents, triggering and ending the inflammatory process, and secreting cytokines and growth factors to recruit and activate other cells involved in the repair process.¹²

One of the limitations of our study is that only one pro-inflammatory cytokine, TNF- α , was used to assess wound age in sharp trauma. Estimating wound age is complex and multifactorial. By using a combination of several parameters such as other pro-inflammatory cytokines, it can reduce errors in the estimation of wound age. Because there are no fixed parameters or methods for assessing wound age, there is a need for systematic and specific criteria to identify molecular markers.

The results obtained from experimental animal studies cannot be easily applied to humans. Although the genetic identity between humans and mice is more than 95%, the application of results from experimental animal studies lacks definitive evidence support for humans. Thus, the problem of transferring the results obtained in animal models to humans and effectively utilizing the large amount of data generated to determine wound age remains unresolved. Significant differences in wound healing in the skin

between humans and mice should not prevent the use of experimental animals. Despite many morphofunctional, immune, and genetic differences, mice have greatly contributed to the field of knowledge about wound healing in humans.

CONCLUSION

There was no significant relationship between TNF- levels and the number of neutrophils with wound age in wounds caused by sharp trauma. The use of TNF- and neutrophils as markers in determining wound age still need to be considered.

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CONFLICT OF INTEREST

The author reports no conflicts of interest in this work.

AUTHOR CONTRIBUTION

All authors were equally involved in making concepts and planning the research, data collection, calculating the experimental data, performing analysis, data analysis, and critical revision of the manuscript.

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