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# Comparison of ELISA Between Pro-Inflammatory Cytokine (IL-1β, IL-6 and TNF- α) Levels Induced by Blunt Trauma Without Damage to The Skin in *Rattus Novergicus*

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Trauma without damage to the skin, IL-1β, IL-6, TNF-α, *Rattus novergicus.*

**ABSTRACT**

Blunt force injuries is the most common type of violence in physical abuse. There were several cases of physical abuse due to blunt force where the victim who was traumatized by blunt force did not have any damage to the skin. Factors that affect the occurrence of trauma without a damage to the skin include the location of contact between the trauma and the body region, pressure (force), the time lag between the victim experiencing trauma and the time to see a doctor, the color of a person's skin, blood pressure, and the thickness of skin tissue. This can be an obstacle for doctors to find physical evidence due to trauma to be written into the medico-legal reports. Blunt trauma that occurs in a person's body will trigger pro-inflammatory mediators such as Interleukin 1-β (IL-1β), Interleukin-6 (IL-6) and Tumor Necrosis Factor-α (TNF-α). This study is an experimental study using animal models. The samples were divided into the control group and the group which the blood serum was taken 1 and 6 hours after being blunt traumatized. The results show there was no significant difference in IL-1β (p=0.161) and TNF-α (p=0.678) levels and there was a significant difference in IL-6 levels (p=0.004) between the control and intervention groups. There was a significant difference on IL-6 cytokine levels between the control group and the intervention groups. The results indicate that pro-inflammatory cytokine IL-6 has the potential to become new examination procedures in cases of trauma without damage to the skin.

## 1. Introduction

Blunt force injuries is the most common type of violence in physical abuse. There were several cases of physical abuse due to blunt force where the victim who was traumatized by blunt force did not have any damage to the skin [1]. Factors that affect the occurrence of trauma without a damage to the skin include the location of contact between the trauma and the body region, pressure (force), the time lag between the victim experiencing trauma and the time to see a doctor, the color of a person's skin, blood pressure, and the thickness of skin tissue [2- 8].

Bruises are the most common type of injury found in cases of physical abuse [9]. Bruising results from the rupture of capillaries or veins beneath the surface of the skin without damaging the integrity of the skin tissue. Trauma (physical violence) that occurs to a person's body does not always cause skin discoloration in the form of bruising (trauma without damage to the skin) [10], [11].

Research related to the appearance of pro-inflammatory mediator such as Interleukin 1-β (IL-1β), Interleukin 6 (IL-6) and Tumor Necrosis Factor- α (TNF- α) in body tissue that have been wounded has been widely studied before, including research by [12] who examined the appearance of pro-inflammatory mediators in the form of Interleukin 1-β (IL-1β), Interleukin-6 (IL-6) and Tumor Necrosis Factor-α (TNF-α) through immunohistochemical examinations carried out through microscopic observations of wounded human skin.

There is also research conducted by [13] who observed the migration of mononuclear and polymorphonuclear cells and several histochemical enzymes such as alkaline phosphatase, acid phosphatase, amino-peptidase, esterase, and ATPase. Furthermore, the research of Kostadinova-Petrova et al., which divides changes in tissue and cell structure in the skin that has a bruise into 2 groups. The first group is fresh bruises where the age of the bruise is under 24 hours post-trauma and the second group is a group of bruises aged 3 - 7 days. However, until now there has not been a single study that examines the pro-inflammatory cytokine response in trauma without damage to the skin.

This study aimed to evaluate the comparison between IL-1β, IL-6 and TNF-α levels in blood serum induced by blunt trauma without damage to the skin throught ELISA examination. Hopefully, this research can provide new examination procedures through the changes in pro-inflammatory cytokine levels due to blunt trauma without damage to the skin, which is an important issue in the forensic field.

## 2. Material and Methods

### *2.1 Study design*

This study design was experimental research with a posttest-only group design. Totally, 30 rats that used in this study were divided into three groups. Each group consists of 10 rats i.e. control group, 1 hour and 6 hours after being traumatized. This study followed the Helsinki Declaration guideline and was approved by the Ethical Committee of Health Research Medical Faculty of Universitas Riau with registered number: B/163/UN19.5.1.1.8/UEPKK/2022.

### *2.2 Animal model*

Male albino strain rats (*Rattus novergicus*) aged 8-9 weeks and weighing above 200 grams were included in this study. The exclusion criteria were animals with stress, illness and death before this study began. Rats were kept in open, humid and well-ventilated cages with 12 hours life / light cycle. Food and drink were given ad libitum.

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***2.3 Experimental procedure***

The rats in this study were traumatized to make blunt injuries by dropping a metal object weighing 324 grams at a height of 45 cm on musculus gracilis dextra. Blood serum samples were taken from the intracardiac. Blood serum was taken after 1 hour and 6 hours in traumatized groups and immediately in the control group. Blood serum was collected to measure IL-1β, IL-6 and TNF-α levels by ELISA method using IL-1β, IL-6 and TNF-α detection kit (EliKine™).

***2.4 Data analysis***

Data were collected and analyzed statistically using the *ANOVA* parametric test to compare IL-1β levels and the *Kruskall-wallis* non parametric test to compare IL-6 and TNF-α levels in each group. A p-value < 0.05 was considered statistically significant.

**3. Finding and Discussions**

In this study, it was found that the highest levels of IL-1β from blood samples were found in the control group with a mean value of 0.143 ± 0,028 compare to IL-1β from blood serum samples in 1 hour post intervention group and 6 hours post intervention group with each mean value of 0.126 ± 0,013 and 0.127 ± 0,019. A similar phenomenon related to the highest levels in the control group also appeared in TNF-α levels, where the control group with a median value of 0.097 (0.0762 - 0.2011) was higher than TNF-α levels 1 hour post intervention group and 6 hours post intervention group with values of 0.94 (0.0548 - 0.1043) and 0.091 (0.0741 - 0.2495), respectively.

Meanwhile, a different phenomenon was found in IL-6 levels, where the lowest value was found in the control group with a value of 0.051 (0.0470 - 0.0541) compared to 1 hour post intervention group and 6 hours post intervention group with values of 0.053 (0.0515 - 0.0862) and 0.054 (0.0528 - 0.0562) respectively.

Based on *ANOVA* test analysis, it was found that there was no relationship between IL-1β levels in blood serum between the control group and the intervention groups. Statistical test results using *Kruskall-wallis* on TNF-α levels between the control and intervention groups also showed no significant relationship. However, for IL-6 levels in blood serum samples, the *Kruskall-wallis* test showed a significant relationship between the control and intervention groups.

Evidence related to victims who experience trauma without damage to the skin is important to note. This is because evidence of injuries can be used as medical testimony that can be outlined in a Medico-legal reports. This is a problem for victims who experience trauma without injury because there is no evidence of physical violence visible on the surface of the body to prove the persecution. The aim of this research is to obtain an alternative supporting examination for cases of trauma without damage to the skin, so that it can become medical evidence that can be written into the Medico-legal reports.

Previous studies have discussed the impact of trauma on increasing levels of pro-inflammatory cytokines, but these studies have focused on trauma that causes open wounds or trauma that causes visible imprints on the skin surface. Our study focuses on the impact of trauma without damage to the skin on the levels of pro-inflammatory cytokines, particularly IL-1β, IL-6 and TNF-α.

In our study, we found lower levels of IL-1β and TNF-α in the intervention group compared to the control group with no significant difference between the two groups. However, IL-6 levels were higher in the intervention group compared to the control group. This indicates an increase in IL-6 levels over time, with

no significant difference between the two groups.

Previous research conducted by [14], using a sample of 50 human wounds to determine the potential of cytokines as markers of determining the age of the wound by ELISA method, showed that there was no statistically significant difference in IL-1β levels in both the test group and the control group. Based on the observations made, it was found that IL-1β levels in the wound area were lower than those in the no-wound group at <30 minutes (33.84 vs 42.88). However, in the same study, with observations on wounds that had occurred 6-12 hours, a significant difference was found between the wound group and the control group ($p = 0.043$) with IL-1β levels in the wound group lower than the control group (28.59 vs 103.05). Research by [14] has similarities with the results of our study, where it was found that between the intervention group compared to the control group there were lower IL-1β levels in the intervention group, which was not statistically significant ($p = 0.147$).

There is also research conducted by [15] by analyzing open wounds on 24 cadavers by taking samples on skin that is not injured and on skin that has open wounds (vital wounds) using needle-puncture. In Peyron's research, IL-1β levels were measured in various groups using the multiplex immunoassay method. The samples were divided into three groups (vital wound, post-mortem wound and control) and the highest IL-1β levels were obtained in the vital wound group followed by the post-mortem wound and control groups (58.62 vs 30.00 vs 19.82). In this study, it was found that there was an increase in IL-1β levels compared to the control group, so in the end the conclusion of this study was that cytokines can play a relevant role in determining the age of the wound.

This research by Peyron is not in line with our study, this may occur because the wounds analyzed by Peyron came from open wounds that have real footprints. The next difference is in the different cytokine analysis methods, where our study used the ELISA principle while Peyron's study used the multiplex sandwich immunoassay method. The difference in the multiplex sandwich immunoassay method lies in the shorter duration of processing time, the use of a smaller number of serum and supernatant samples compared to ELISA [15].

Explicitly, no one has compared the difference in cytokine levels in open wounds compared to closed wounds (bruises). However, differences are possible because in open wounds the increase in pro-inflammatory cytokine levels can be triggered by factors other than trauma, namely external exposure factors such as exposure to bacteria [16].

In our study, the phenomenon of higher cytokine levels in the control group compared to the intervention group, accompanied by no significant difference between the control and intervention groups, was also found in the examination of TNF-α cytokine levels.

The results of our study are inversely proportional to the study conducted by Birincioğlu İ et al. in 2016. Birincioğlu used 50 samples of wounds on the human body to determine the potential of cytokines as markers in determining the age of wounds by ELISA method. The study showed a statistically significant increase in TNF-α levels in the intervention group <30 minutes after wound exposure compared to the control group (7.49 vs 2.88). TNF-α will remain at high levels but not statistically significant after >30 minutes [14]. This difference occurs because in Birincioğlu's study, sampling was conducted in humans while our study was conducted in Rattus norvegicus where there is a difference in the time of the wound healing process in humans compared to mice (life cycle of mice / rats between humans), where 18 seconds of human time is equivalent to 1 hour of mouse time. Another factor that causes the difference in results is

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that Birincioğlu's study used samples from various types of wounds (firearms, sharp weapons and blunt weapons) while this study only used samples of bruises caused by blunt violence. This wound type factor is a differentiator because open wounds have greater force and exposure, potentially leading to higher cytokine levels than in trauma without damage to the skin.

Our study is also inversely proportional to El-Zahed's 2020 study on the role of TNF-α in determining the age of wounds in human cadavers by histopathology and immunohistochemistry. El-Zahed's study used 20 autopsy cases (16 cases of stab wounds with wound ages varying from 30 to 300 minutes and 4 cases without wounds as a control group) which were divided into: group I (wound time ≤ 30 minutes), group II (wound time 31 - 60 minutes), group III (wound time 61 - 120 minutes), group IV (wound time > 120 minutes) and control. The group was further divided into 2 subgroups, namely subgroup A (far from the injury site) and subgroup B (right at the injury site). The results showed that the percentage of TNF-α area was lower in the control group than the other 4 groups with group II showing significantly higher results than group I and the control group. Statistically significant high results in TNF-α percentage area were detected in groups III and IV when compared to the control group, group I and group II [17].

In the comparison between the wounded group and the group without wounds in the same group, it was found that there was no significant difference in the ≤ 30 minutes group and the 31 - 60 minutes group, but showed a significant increase in the TNF-α percentage area in the wounded group in the 61 - 120 minutes and > 120 minutes groups [17]. The difference between our study and El-Zahed's study may occur due to differences in the method of detecting TNF-α, where in our study we used ELISA and El-Zahed used immunohistochemical methods.

In our study, there was a difference in median IL-6 levels between the 6-hour post-intervention group and the control group of 0.003 and a difference in median IL-6 levels between the 1-hour post-intervention group and the control group of 0.002. Statistical test results between the test groups with IL-6 levels using *Kruskall-wallis* showed there was a statistically significant difference (p = 0.004). These results provide a different picture from the results of the two previous cytokines, namely IL-1β with TNF-α.

The results in our study show similar results to the study by [14] in a group of bodies that were injured for a period of > 18 hours. Similarities occurred in the increase in IL-6 levels with increasing time and there was a significant difference between the control group and the intervention group. Research by [14] used 50 human wound samples to determine the potential of IL-6 as a marker in determining wound age using the ELISA method. The study compared IL-6 levels in body parts that had wounds with body parts that did not have wounds (control) and compared based on time periods <30 minutes, 30 minutes - 1 hour, 2 hours - 4 hours, 6 - 12 hours, and > 18 hours. Only at >18 hours there was a significant difference with higher IL-6 levels in the wound condition compared to the control and statistically significant (302.60 vs 144.49, p = 0.018). While in other time periods there was no statistically significant difference between wound and control conditions, it should be noted that in theory IL-6 levels in wound conditions will always be higher than controls.

Similar results also occurred in the study of [15] where he analyzed open wounds in 24 cadavers by taking samples of uninjured skin and skin samples that had open wounds using needle-puncture. The results of the study showed higher levels of IL-6 in the wound compared to the control sample (241.2 pg/mL vs 17.19 pg/mL). IL-1β levels, IL-6 levels and TNF-α levels in blood serum samples data are provided in the tables below.

**Table 1.** IL-1β levels in blood serum samples

| Groups | IL-1β levels (pg/ml) Mean ± SD | p* |
|---|---|---|
| **Control** | 0.143 ± 0,028 | |
| **1 Hour Post Intervention** | 0.126 ± 0,013 | 0.161 |
| **6 Hour Post Intervention** | 0.127 ± 0,019 | |

**ANOVA* test, Significant p < 0,05.

**Table 2.** IL-6 levels in blood serum samples

| Groups | IL-6 levels (pg/ml) Median (Min – Max) | p* |
|---|---|---|
| **Control** | 0.051 (0.0470 – 0.0541) | |
| **1 Hour Post Intervention** | 0.053 (0.0515 – 0.0862) | 0.004 |
| **6 Hour Post Intervention** | 0.054 (0.0528 – 0.0562) | |

*Kruskall-wallis test, Significant p < 0,05.

**Table 3.** TNF-α levels in blood serum samples

| Groups | TNF-*α* levels (pg/ml) Median (Min-Max) | p* |
|---|---|---|
| **Control** | 0.097 (0.0762 – 0.2011) | |
| **1 Hour Post Intervention** | 0.094(0.0548 – 0.1043) | 0.678 |
| **6 Hour Post Intevention** | 0.091 (0.0741 – 0.2495) | |

* *Kruskall-wallis* test, Significant p < 0,05.

## 4. Conclusion

The results of this study show that there was no significant difference in the levels of cytokines IL-1β and TNF-α between the control group and the intervention group that was exposed to loads that caused trauma without injury. In fact, there is a tendency to decrease the levels of both cytokines in each examination panel as time increases.

A different phenomenon was shown in the results of the IL-6 cytokine examination, where there was a significant difference between the control group and the intervention group exposed to the load to cause trauma without damage to the skin. In fact, there was a tendency for the cytokine levels to increase in each examination panel over time. The results of this examination indicate that the pro-inflammatory cytokine IL-6 has the potential to become a supporting examination modality in cases of trauma without damage to the skin.

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