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The effect of duration of wound skin tissue on MDA, TNF-α, IL-6, Caspase 3, VEGF levels, and granulation tissue thickness in the white rat (Rattus novergicus)

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ABSTRACT

Introduction: Wound is the result of physical trauma that severs skin continuities. The damage typically causes an inflammatory reaction marked by increase ROS and tissue inflammation. However, this process is still needed to be further investigated. Therefore, this study aimed to evaluate plasma MDA, IL-6, TNF-α, Caspase-3 and VEGF levels, and the granulation tissue thickness at different time points during the wound healing process.

Methods: An experimental post-test only control group study was conducted using white male rats (Rattus Norvegicus) and the rats were wounded and assessed on day 5 (K1), day 10 (K2), and day 15 (K3). The level of plasma MDA, IL-6, TNF-α, Caspase-3 and VEGF level was assessed as well as the granulation tissue thickness.

Results: The OneWay ANOVA test results showed significant differences in plasma MDA, IL-6, TNF-α, Caspase-3 and VEGF levels (P <0.05). The Tukey HSD test results showed significant differences between (P <0.05) K1 with K2, K1 and K3 and K2 with K3 on MDA plasma, IL-6, TNF-α, Caspase-3 and VEGF. However, the granulation tissue thickness was only slightly different between groups and was not significant.

Conclusion: The inflammation and angiogenesis were steadily increased overtime during the wound healing process while oxidative stress and cell death were decreasing. However, none of those factors were related to granulation thickness.

Keywords: Wound healing, MDA, IL-6, TNF-α, Caspase-3, VEGF, Granulation Tissue

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INTRODUCTION

The wound is the result of physical trauma that severs skin tissues. Various factors affect the cellular and molecular processes after injury, including the wound's type, size, and depth. Meanwhile, the phases of wound healing generally involve platelet aggregation, fibrin formation, angiogenesis and re-epithelialization. Acute wound healing shows a linear process, whereas, in chronic wounds, the process is not synchronous, with several parts being in different phases at the same time.[1] Current evidence showed that oxidative stress might also influence the wound healing process, in which it needs to be balanced.[2] Non-radical metabolites such as hydrogen peroxide have the potential to be harmful at an excessive level, whereas radical metabolites are potentially damaging through lipid peroxidation, protein modification, and DNA modification.[3]

ROS plays an essential role in wound healing through complex mechanisms, including migration, adhesion, proliferation, neovascularization, remodeling, and apoptosis. The role of ROS in the molecular aspect, among others, is to induce the expression of pro-inflammatory cytokines and directly or indirectly become the source of damage toward fibroblasts and keratinocytes, both structurally and functionally. A balanced ROS level is very crucial in determining the course of wound healing outcomes. For example, high ROS levels can hamper angiogenesis by altering the level of VEGF, TNF-α, IL-6 and TGF-β. These processes will then affect the formation of granulation tissue on days 4-7.[4] Altered angiogenesis will affect the rate and thickness of granulation tissue formed because the cellular migration, nutrient distribution, and oxygenation depend on angiogenesis.[5] However, the relationship between ROS with angiogenesis, granulation tissue formation, and wound healing is still poorly understood. Therefore, according to the previous description, this study was aimed to examine the relationship between wound care duration on the levels of malondialdehyde (MDA), TNF-α, IL-6, Caspase 3, VEGF, and the granulation tissue thickness.

METHODS

Study Design and Animal

This study used experimental post-test only control group design and white male rats *(Rattus norvegicus)* as the sample. The rats aged between 3-4 months and had 150–300 grams in body weight obtained from the Faculty of Veterinary Medicine, Gajah Mada University. The procedures and sample examinations were carried out in PAU UGM Yogyakarta, while immunohistochemical observations were carried out at Anatomical Pathology, Faculty of Medicine, Sebelas Maret University Surakarta by pathologic experts.

According to the sample size formula, a minimum number of samples required in each group were five white male rats. By adding one more rat per group, this study needed 24 male rats divided into 4 groups. The rats were selected using several inclusion criteria, including white male rats with glowing eyes, no dull hair, physically active with a good appetite, aged between 3-4 months, and weighing between 150-300 grams. The rats were fed standard BR I food with the amount adjusted for the average body weight. The exclusion criterion was white male rats that died during the study. This study protocol had been approved by the Indonesian Institutional Review Board (Protocol Number 746/VI/HERC/2020).

Wounding Procedure and Observation

Before the wounding procedure, the mice were acclimatized for five days. The day before the wound was made, the animals had their dorsal smoothly shaved and then cleaned with 70% alcohol. A square incision was made at 2x2 cm in size and then the wound was treated. The animals were divided into four groups, namely group 1 (K1) without treatment or negative control group, group 2 (K2) with the wound not being treated for five days, group 3 (K3) with the wound not being treated for ten days and group 4 (K4) with the wound not treated for 15 days.

Figure 1. Wounding procedure on the rat's dorsal skin

Blood MDA, TNFα, IL-6, Caspase-3 and VEGF Assessment

The retroorbital blood was drawn on day 0 (group 1), day 5 (group 2), day 10 (group 3) and day 15 (group 4) to check the levels of MDA, TNFα, IL-6, Caspase-3 and VEGF. MDA level was determined by the thiobarbituric acid reactive substance (TBARS) method, while TNFα, IL-6, Caspase-3 and VEGF were examined using ELISA.

Animal termination was carried out on day 5 (group 2), day 10 (group 3), and day 15 (group 4). The wound tissue and some of the surrounding healthy tissues were taken and completely immersed using a combined solution of 10% formalin with Phosphate Buffer Saline (PBS) in a urine pot to fix the tissues. Each urine pot was labeled with the name of the group as identification. The paraffin block and IHC staining were used to assess the height of the granulation tissue.

The granulation tissue height was then evaluated using the lowest power (10x magnification) to determine the most intense staining area using the HE staining technique. Then the network height was calculated on a micrometer scale.

Statistical Analysis

Data analysis was performed using *SPSS version 24 for Windows* and data presentation was performed using Microsoft Office 2010. All data were tested for normality test (*Shapiro-Wilk test)* to determine further analysis. If the data distribution was normal, One Way Anova was used to compare the sample of groups. Otherwise, the alternative statistical test is performed if the data distribution was not normal (Kruskal-Wallis test).

RESULTS

Plasma MDA Level Assessment

According to the result of the plasma MDA examination, all of the wounded groups (II-IV) experienced increased MDA levels compared to the control group. The highest mean MDA was observed in group II (5 days post wounding) and the concentration was lower in two other groups. Shapiro-Wilk test showed a non-significant result in which the data was then analyzed using ANOVA. The ANOVA test showed a significant value, which indicated that the differences between groups were significant (Table 1).

Table 1. Plasma MDA levels in rat groups

Group	N	Mean of Plasma MDA ± SD (nmol/mL)	P-Value*
I (K1)	6	1.38 ± 0.227	< 0.05
II (K2)	6	8.99 ± 0.004	
III (K3)	6	8.46 ± 0.006	
IV (K4)	6	6.44 ± 0.005	

*P-Value from ANOVA

Table 2. The post-hoc analysis of on plasma MDA level between groups

Group	Sig.	Difference
K1 with K2	0.000	Significant
K1 with K3	0.000	Significant
K1 with K4	0.000	Significant
K2 with K3	0.000	Significant
K2 with K4	0.000	Significant
K3 with K4	0.000	Significant

Table 3. Plasma IL-6 Levels in Mice Group

Group	N	Mean of IL-6 ± SD (pg/ml)	P-Value*
I (K1)	6	70.82 ± 2.870	
II (K2)	6	106.18 ± 0.270	< 0.05
III (K3)	6	92.23 ± 0.350	
IV (K4)	6	79.12 ± 0.320	

*P-Value from ANOVA

Table 4. The post-hoc analysis of plasma IL-6 level between groups

Group	Sig.	Difference
K1 with K2	0.000	Significant
K1 with K3	0.000	Significant
K1 with K4	0.000	Significant
K2 with K3	0.000	Significant
K2 with K4	0.000	Significant
K3 with K4	0.000	Significant

To pinpointed the significant differences between groups, a Post-hoc Tukey analysis was conducted. The analysis showed that all comparisons were significantly different. This result indicated that the plasma MDA level increased after wounding and continuously decreased over time (Table 2).

Plasma IL-6 Level Assessment

According to the plasma IL-6 examination result, all of the wounded groups (II-IV) experienced an increased level of IL-6 compared to the control group. The highest mean IL-6 was observed in group II (5 days post wounding) (106 pg/ml) and the concentration was decreased in two other groups. Shapiro-Wilk test showed a non-significant result in which the data was then analyzed using ANOVA. The ANOVA test showed a significant value, which indicated that the differences between groups were significant (table 3).

To pinpointed the significant differences between groups, a Post-hoc Tukey analysis was conducted. The analysis showed that all comparisons were significantly different. This result indicated that the plasma IL-6 level increased after wounding and continuously decreased over time (Table 4).

Plasma TNF-α Level Assessment

According to the result of the plasma TNF-α examination, all of the wounded groups (II-IV) experienced an increased level of TNF-α compared to the control group. The highest mean TNF-α was observed in group II (5 days post wounding) (14.15±0.230 pg/ml), and the concentration was decreased in two other groups. Shapiro-Wilk test showed a non-significant result in which the data was then analyzed using ANOVA. The ANOVA test showed a significant value, which indicated that the differences between groups were significant (table 5).

To pinpointed the significant differences between groups, a Post-hoc Tukey analysis was conducted. The analysis showed that all comparisons were significantly different. This result indicated that the plasma TNF-α level increased after wounding and continuously decreased over time (Table 6).

Plasma Caspase-3 Level Assessment

According to the plasma Caspase-3 examination result, all of the wounded groups (II-IV) experienced an increased level of Caspase-3 compared to the control group. The highest mean Caspase-3 was observed in group II (5 days post wounding) (7.14±0.208 pg/ml) and the concentration was decreased in two other groups. Shapiro-Wilk test showed a non-significant result in which the data was then analyzed using ANOVA. The ANOVA test showed a significant value, which indicated that the differences between groups were significant (table 7).

To pinpointed the significant differences between groups, a Post-hoc Tukey analysis was conducted. The analysis showed that all comparisons were significantly different. This result indicated that the plasma Caspase-3 level increased after wounding and continuously decreased over time (Table 8).

Plasma VEGF Level Assessment

According to the plasma Caspase-3 examination result, all of the wounded groups (II-IV) experienced an increased level of Caspase-3 compared to the control group. The highest mean Caspase-3 was

Table 5. Plasma TNF-α Levels in rat group

Group	N	Mean of TNF-α ± SD	P-Value*
I (K1)	6	6.21 ± 0.230	<0.05
II (K2)	6	14.15 ± 0.230	
III (K3)	6	9.15 ± 0.120	
IV (K4)	6	7.18 ± 0.350	

*P-Value from ANOVA

Table 6. The post-hoc analysis of on plasma TNF-α level between groups

Group	Sig.	Difference
K1 with K2	0.000	Significant
K1 with K3	0.000	Significant
K1 with K4	0.000	Significant
K2 with K3	0.023	Significant
K2 with K4	0.000	Significant
K3 with K4	0.000	Significant

Table 7. Plasma Caspase-3 levels in rat groups

Group	N	Mean of Caspase-3±SD (pg/ml)	P-Value*
I (K1)	6	3.87 ± 0.054	< 0.05
II (K2)	6	7.14 ± 0.208	
III (K2)	6	6.06 ± 0.160	
IV (K3)	6	4.86 ± 0.700	

*P-Value from ANOVA

Table 8. The post-hoc analysis of on plasma Caspase-3 level between groups

Group	Sig.	Difference
K1 with K2	0.000	Significant
K1 with K3	0.000	Significant
K1 with K4	0.000	Significant
K2 with K3	0.000	Significant
K2 with K4	0.000	Significant
K3 with K4	0.000	Significant

observed in group II (5 days post wounding) (7.14±0.208 pg/ml) and the concentration was decreased in two other groups. Shapiro-Wilk test showed a non-significant result in which the data was then analyzed using ANOVA. The ANOVA test showed a significant value, which indicated that the differences between groups were significant (table 9).

To pinpointed the significant differences between groups, a Post-hoc Tukey analysis was conducted. The analysis showed that all comparisons were significantly different, except between K1 and K4. This result indicated that the plasma VEGF level decreased after wounding but increased over time until it reached the pre-wounding level on day 15 and continuously decreased over time (Table 10).

Granulation Tissue Thickness Assessment

According to the tissue assessment, it appeared that the granulation tissue thickness was initially increased from day 5 (K1) to day 10 (K2) but then decreasing at day 15 (K3). Statistical analysis revealed that there was no significant difference between the thickness of granulation tissue between study groups.

DISCUSSION

The findings in this study confirmed and supported current knowledge in the wound healing process, assessed from the oxidative stress, inflammation, cell death, and angiogenic points of view. Our study clearly showed that the MDA level was sharply increased at day five post-wounding and steadily decreasing afterward. The MDA is formed as the byproduct of oxidative stress reaction and, therefore, can reflect the oxidative status of the individuals.[6] The ROS might be produced by the damaged cells or the invading immune cells that combat the infection within the wounded tissues or the immune cells clearing the lytic cells. This finding is consistent with the acute nature of the wound and inflammation.

During the inflammatory phase of the wound healing process, immune cells invaded the tissue and became activated, marked by increased pro-inflammatory cytokines and ROS expression. The accumulation of Reactive Oxygen Species (ROS) induces oxidative stress in tissues and interact with lipid molecules, which cause lipid peroxidation. Lipid peroxidation produces lipid hydroperoxides (LOOH) as the main product, while the byproducts include malondialdehyde (MDA) and 4-HNE.[7] Therefore, the longer of duration the wound healing process, the higher the number of damaged cells as well as inflammation and ROS, produced both by damaged cells and immune cells. This phenomenon will then reflected as the increased level of plasma MDA.[8]

The concentration of IL-6 and TNF-α can also reflect the inflammatory and oxidative status of wounded individuals. IL-6 production is induced by ROS and endoplasmic reticulum stress, while TNF-α is the primary cytokine in acute phase inflammation. IL-6 is a mediator that plays a role in the inflammatory process, immune response, and hematopoiesis. Our finding is consistent with several other studies, which also showed increased IL-6 levels in the inflammatory phase of wound healing[6]. Some of those studies also showed an increased level of TNF-α, which then, in conjunction with IL-6, further reinforces the inflammatory reaction.[9]

On the other hand, cell survival is also an essential aspect of wound healing. The level of

Table 9. Plasma VEGF Levels in rat groups

Group	N	Mean of VEGF ± SD (pg/ml)	P-Value*
I (K1)	6	33.78 ± 1.890	<0.05
II (K2)	6	23.13 ± 1.297	
III (K3)	6	27.05 ± 1.130	
IV (K4)	6	32.09 ± 1.110	

*P-Value from ANOVA

Table 10. The post-hoc analysis of on plasma VEGF level between groups

Group	Sig.	Difference
K1 with K2	0.000	Significant
K1 with K3	0.000	Significant
K1 with K4	0.185	Not Significant
K2 with K3	0.000	Significant
K2 with K4	0.000	Significant
K3 with K4	0.000	Significant

Table 11. Granulation tissue thickness in all test groups

Group	N	Granulation Tissue Thickness	P-Value*
I (K1)	6	0	<0.33
II (K2)	6	825.5 ± 228.39	
III (K3)	6	864.75 ± 265.78	
IV (K4)	6	802.5 ± 149.27	

Caspases reflects the rate of apoptosis of the injured cells and one of them is Caspase-3. The activation of caspase-3 is closely associated with increased intracellular reactive oxygen species (ROS). Increased ROS results from overactivity of the mitochondria, leading to intercellular oxidative stress. Simultaneously, this excess cellular metabolic activity forces the synthesis of excess protein in the endoplasmic reticulum (ER), resulting in ER stress. Cellular metabolic stress also induces neuroinflammation by activating pro-inflammatory cytokines NF-Kb, AP-1, and X-box binding protein (XBP1). NF-Kb directly induces apoptosis, which will be mediated by Caspase-3. Caspase-3 is an apoptotic regulatory protein in neural precursor cells (NPCs) and post-mitotic neurons.[10]

However, ROS also had other essential functions, which is the induction of angiogenesis. The endogenous ROS, mainly involved in angiogenesis, is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase from NOX and electron transport chain reactions mitochondria. NADPH oxidase can be activated by various growth factors, including VEGF, angiopoietin-1, ischemia, and hypoxia. Conversely, ROS derived from NADPH oxidase plays an essential role in the autophosphorylation process of VEGFR-2.[11]

The increase of granulation tissue thickness on day 5 and 10 was mainly due to the higher cellular proliferation process during this phase, which corresponds to its name: proliferation phase. This phase usually lasts for 48 hours after the injury. On the other hand, on the 15th day, the wound enters the remodeling phase, so a slight decrease in the granulation thickness. This phase lasts from the 14th day until one year. It involves a significant reduction in cellularity, which resulted from apoptosis of residual inflammatory cells and myofibroblasts as well as neovascular regression. The remodeling phase is characterized by wound contraction and collagen remodeling.[12] However, the wound gradually gets stronger over time and its tensile strength increases rapidly.[13]

CONCLUSION

In conclusion, it was clear that the level of MDA, TNF-α, and IL-6 increased overtime until day 15 post-wounding, while the Caspase-3 level had the opposite trend, which was decreased over time as the wound healing process progressed. Meanwhile, the level of VEGF initially dropped but then increased over time as the wound healing progressed. Finally, the granulation tissue only slightly decreased at the end of the study, which may correspond to the scar's contraction.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this study was reported.

ETHIC APPROVAL

This study protocol had been approved by the Indonesian Institutional Review Board (Protocol Number 746/VI/HERC/2020).

AUTHOR CONTRIBUTION

All authors contributed equally in the writing process and revising this article.

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