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## **ABSTRACT**

**Background:** Radiation dermatitis is the most common adverse effect of external radiation. Ozonated aloe vera prompted good tissue healing. This study aimed to investigate the effect of ozonated Aloe vera for improving healing response in *Sprague Dawley* rats suffering dermatitis radiation, regarding TNF- $\alpha$  expression and neutrophil lymphocyte ratio histopathologically.

**Methods** : Thirty-six male Sprague Dawley rats were randomly divided into groups. There were group K (negative control), P1 (positive control), P2 (Aloe vera), and P3-P5 (graded dose of ozonated aloe vera). The intervention conducted in 7 days. TNF- $\alpha$  expression and neutrophil-lymphocyte ratio observed under immunohistochemistry staining and HE staining consecutively.

**Result** : There were significant differences in neutrophil-lymphocyte ratio between groups in which the highest NLR average ( $1.15 \pm 0.77$ ) in negative control group and the lowest NLR average ( $0.02 \pm 0.04$ ) in Hydrocortisone group. Significant differences in TNF- $\alpha$  were analyzed between groups with the highest TNF- $\alpha$  average ( $6.76 \pm 0.41$ ) in control group and the lowest TNF- $\alpha$  average ( $3.72 \pm 1.13$ ) in Hydrocortisone group.

**Conclusion** : Ozonated aloe vera application was able to increase the healing process in *Sprague Dawley* rats suffered from radiation dermatitis by decreasing the amount of TNF- $\alpha$  expression and NLR.

**Keywords:** *Radiation dermatitis, ozonated aloe vera, radiation dermatitis, TNF- $\alpha$  expression, neutrophil-lymphocyte ratio*

## BACKGROUND

Radiation dermatitis is the most common adverse effect of external radiation. Radiation dermatitis manifests either as acute erythema and desquamation or chronic skin atrophy and telangiectasis. The incidence and severity of radiation dermatitis varies depending on external factors including radiation dose and area of radiation administration.<sup>1-2</sup>

Adverse effects occurred based on the type of radiotherapy and manifest in the treated area affecting both organs and tissues.<sup>3,4</sup> Acute radiation-induced dermatitis or radiation dermatitis affect up to 95% of patients receiving radiotherapy.<sup>5</sup> The clinical manifestations of radiation dermatitis vary from mild erythema of the skin to skin necrosis.<sup>6</sup>

The skin is an organ that continuously divides. Keratinocytes and melanocytes are radiosensitive therefore radiation exposure can affect its division cycle. Repeated radiation exposure leads to decreased time for cell repair. Notably, cell and tissue injury may increase. Inflammatory cell responses may happen in excessive amount that can interfere wound healing by disrupting granulated tissue formation, fibrogenesis, and angiogenesis.<sup>1,2</sup>

Radiotherapy promotes cytokines releases, cell cycle changes, and DNA damages that activate inflammatory responses and oxidative stress reactions. In the early phase of inflammation, pro-inflammatory cytokines such as IL-1, IL-3, IL-5, IL-6, and Tumor Necrosis Factor Alfa (TNF- $\alpha$ ) released and causing eosinophils and neutrophils infiltration that leads to tissue damage. Furthermore, this could trigger a domino effect of the inflammatory response.

4-7

Aloe vera is widely used as a topical therapy for skin tissue inflammation due to its anti-inflammatory and antioxidant properties. Aloe vera can reduce inflammation and accelerate skin healing by inhibiting cyclooxygenase and cytokines that play an important role in inflammatory reactions. Aloe vera also accelerate skin healing and tissue repairing by reducing neutrophil infiltration and platelet aggregation.<sup>8</sup>

Ozonated aloe vera prompts good tissue healing by increasing oxygen supply and healing factors in tissues. It also regulate antioxidant enzymes and gives protection against oxidative reactions.<sup>9-12</sup> Ozone immediately reacts with lipids in the stratum corneum and being absorbed as an antioxidant through the skin.<sup>9</sup> Topical ozonated aloe vera also beneficial for cancer patients suffering radiation dermatitis.<sup>13</sup>

The previous studies of ozonated aloe vera showed a decreased number of neutrophils and wound diameter after being treated with ozonated aloe vera oil. Ozonated aloe vera significantly improving epithelialization process.

This study aims to investigate the effect of ozonated Aloe vera for improving healing response in *Sprague Dawley* rats suffering dermatitis radiation. The results of this study were expected to be beneficial in medical field as an alternative new therapy for radiation dermatitis.

## **MATERIALS AND METHODS**

### *Research design*

This study was an experimental study with post-test only control group design. The samples were divided into groups and the control group did not receive any intervention. The outcome data was recorded after 7 days.

Groups consisted of the negative control group (PK) that did not receive any topical therapy, the positive control group (P1) that receive topical hydrocortisone acetate 2.5% therapy for 7 days, group treatment 2 (P2) that receive Aloe vera therapy for 7 days, group treatment 3 (P3), group treatment 4 (P4), and group treatment 5 (P5) that receive ozonated Aloe vera at a dose of 300 mg/ml, 600 mg/ml, and 1200 mg/ml, respectively. The samples were given a single dose of gamma ray radiation of 7 Gy on the 4x4 cm back area then being observed histopathologically for TNF- $\alpha$  expression and neutrophil-lymphocyte ratio under 400x magnification.

### *Research sample*

*Sprague Dawley* rats was obtained from the Experimental Animal Laboratory, Sultan Agung University, Semarang. The inclusion criteria including male rats aged 2-3 months, body weight  $150\pm 50$  grams after acclimation for a week in individual cages, and no anatomical abnormalities. Exclusion criteria were sick or died rats in time of induction or intervention. Samples were kept at room temperature of  $28.0\pm 2.0^{\circ}\text{C}$  and undergone light lighting for 12 hours (06.00-18.00) and dark at 18.00-06.00. Subjects were being fed ad libitum. The sample number in each group was 6.

Ozonated aloe vera was obtained from the Plasma Research Laboratory of Diponegoro University, resulted from mixing ozone produced by an ozone generator with oil. A magnetic stirrer was used to facilitate mixing process and obtaining ozonated Aloe vera that were ready to be used. Ozone oil samples were tested using a spectrometer or kit dissolved ozone meter. Measurement of ozone expression in ozonated oil was done by potassium iodide titration method.

### *Research variable*

The administration of topical ozonated aloe vera was determined as independent variables. Meanwhile, TNF- $\alpha$  expression and neutrophil-lymphocyte ratio were dependent variables.

### *Research implementation*

The samples of this study were 36 adult male *Sprague Dawley* rats weighing  $150\pm 50$  grams. The samples were kept at room temperature of  $28.0\pm 2.0^{\circ}\text{C}$  undergone light lighting for 12 hours (06.00-18.00) and dark at 18.00-06.00 to reduce the stress levels. The samples were given adequate food. Each sample got a single dose of 7 Gy gamma ray radiation on 4 x 4 cm of back area for 7 days. The radiation process was carried out using a modification of the previous procedure existed.

The samples were divided into 6 treatment groups by simple random sampling. Each

group consisted of 6 samples. After 7 days of intervention, the skin tissues administrated with radiation were taken and processed into histopathological preparations and made into paraffin blocks. Immunohistochemical observation of TNF- $\alpha$  and neutrophil-lymphocyte ratio was done by an anatomical pathologist at Diponegoro University.

#### *Data analysis*

The collected data were processed by editing, coding, tabulation, and entering data. Descriptive data analysis and hypothesis testing were performed. In descriptive analysis, dependent variables were presented in the form of table mean, SD, median and box plot graphics. The One Way Anova test was used to test the hypothesis. The Kruskal Wallis test was chosen when the data abnormally distributed. The Post-Hoc Test was performed to determine the significance differences between groups. The limit of the degree was  $P < 0.05$  with 95% confidence interval.

#### *Research Ethical Requirements*

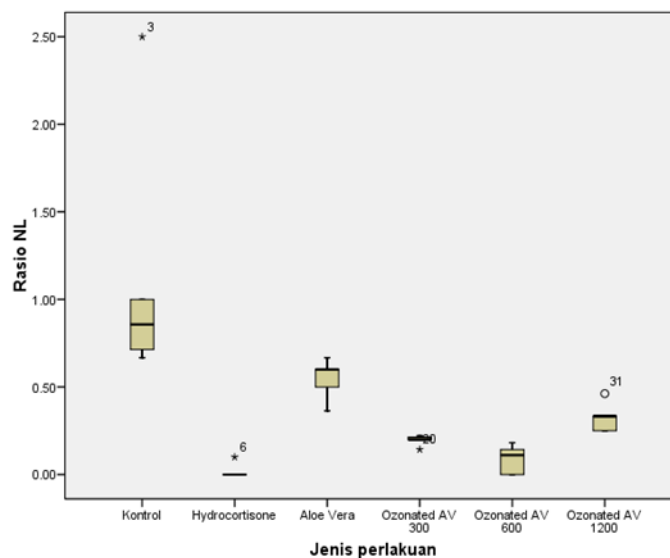
The research was approved by Health Research Ethics Commission of the Faculty of Medicine, Diponegoro University and all samples were being treated and managed according to animal maintenance standards.

## **RESULTS**

**Table 1.** Descriptive and Result of Shapiro-Wilk Normality Test for Neutrophil-lymphocyte Ratio

Treatment	Mean ± SD	Median (min – max)	p
Control	1,15 ± 0,77	0,86 (0,67 – 2,50)	0,011*
Hydrocortisone	0,02 ± 0,04	0,00 (0,00 – 0,10)	0,000*
Aloe vera	0,55 ± 0,12	0,60 (0,36 – 0,67)	0,485
Ozonated AV 300	0,20 ± 0,03	0,20 (0,14 – 0,22)	0,036*
Ozonated AV 600	0,09 ± 0,08	0,11 (0,00 – 0,18)	0,232
Ozonated AV 1200	0,33 ± 0,09	0,33 (0,25 – 0,46)	0,225

\* not normal



**Figure 1.** Boxplot graph of neutrophil- lymphocyte ratio

**Table 2.** Kruskal Wallis Test Result

Treatment	Mean ± SD	P
Control	1,15 ± 0,77	<0,001



Hydrocortisone		0,02 ± 0,04
Aloe vera		0,55 ± 0,12
Ozonated	AV	
300		0,20 ± 0,03
Ozonated	AV	
600		0,09 ± 0,08
Ozonated	AV	
1200		0,33 ± 0,09

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The table of Kruskal Wallis test results showed a significant difference in the NLR based on the type of intervention with p value <0.05. The highest average NLR (1.15 ± 0.77) was in the control group and the lowest NLR average (0.02 ± 0.04) was in the Hydrocortisone group. The Mann Whitney test was put on to find out the differences between groups.

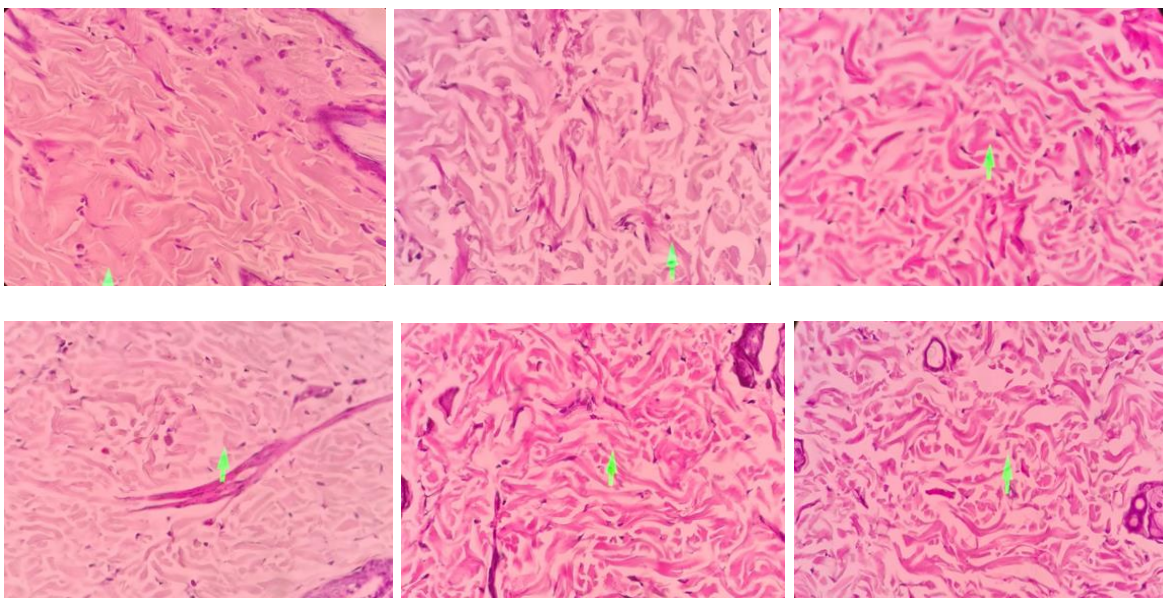
**Table 3.** Mann Whitney Test Result for Neutrophil-lymphocyte Ratio

Treatment		P
I	II	
Control	Hydrocortisone	0,007
	Aloe vera	0,012
	Ozonated AV 300	0,006
	Ozonated AV 600	0,006
	Ozonated AV 1200	0,009
Hydrocortisone	Aloe vera	0,007
	Ozonated AV 300	0,005
	Ozonated AV 600	0,072*

Treatment		P
I	II	
	Ozonated AV 1200	0,007
Aloe vera	Ozonated AV 300	0,006
	Ozonated AV 600	0,006
	Ozonated AV 1200	0,015
Ozonated AV 300	Ozonated AV 600	0,007
	Ozonated AV 1200	0,005
Ozonated AV 600	Ozonated AV 1200	0,006

Significant p < 0,05, \*not significant

The Mann Whitney test was carried out and the result of group being treated with Hydrocortisone against Ozonated AV 600 was not significant, while the other groups results were significant.



**Figure 2.** Histopathological appearance with hematoxylin-eosin staining (400x)

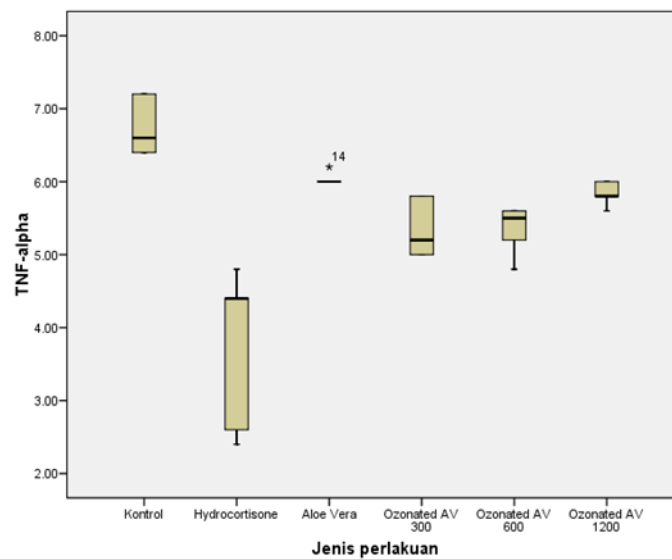
**Notes:** From the top left in a clockwise direction shows the histopathological picture of

groups K, P1, P2, P3, P4, P5

**Table 4.** Descriptive and Result of Shapiro-Wilk Normality Test for TNF- $\alpha$

Treatment	Mean $\pm$ SD	Median (min – max)	P
Control	6,76 $\pm$ 0,41	6,6 (6,4 – 7,2)	0,057
Hydrocortisone	3,72 $\pm$ 1,13	4,4 (2,4 – 4,8)	0,097
Aloe vera	6,04 $\pm$ 0,09	6,0 (6,0 – 6,2)	0,000*
Ozonated AV 300	5,33 $\pm$ 0,39	5,2 (5,0 – 5,8)	0,035*
Ozonated AV 600	5,37 $\pm$ 0,32	5,5 (4,8 – 5,6)	0,070
Ozonated AV 1200	5,83 $\pm$ 0,15	5,8 (5,6 – 6,0)	0,212

\*not normal



**Figure 3.** Boxplot graph of TNF- $\alpha$

**Table 5.** Kruskal Wallis Test Result

Treatment	Mean $\pm$ SD	P
Control	6,76 $\pm$ 0,41	<0,001

Hydrocortisone		3,72 ± 1,13
Aloe vera		6,04 ± 0,09
Ozonated 300	AV	5,33 ± 0,39
Ozonated 600	AV	5,37 ± 0,32
Ozonated 1200	AV	5,83 ± 0,15

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In Kruskal Wallis test results with p value <0.05, there were significant differences in TNF- $\alpha$  depending on the type of intervention with the highest TNF- $\alpha$  average (6.76 ± 0.41) in the control group and the lowest TNF- $\alpha$  average (3.72 ± 1.13) was in the Hydrocortisone group. The Mann Whitney test was put on to find out the differences between groups.

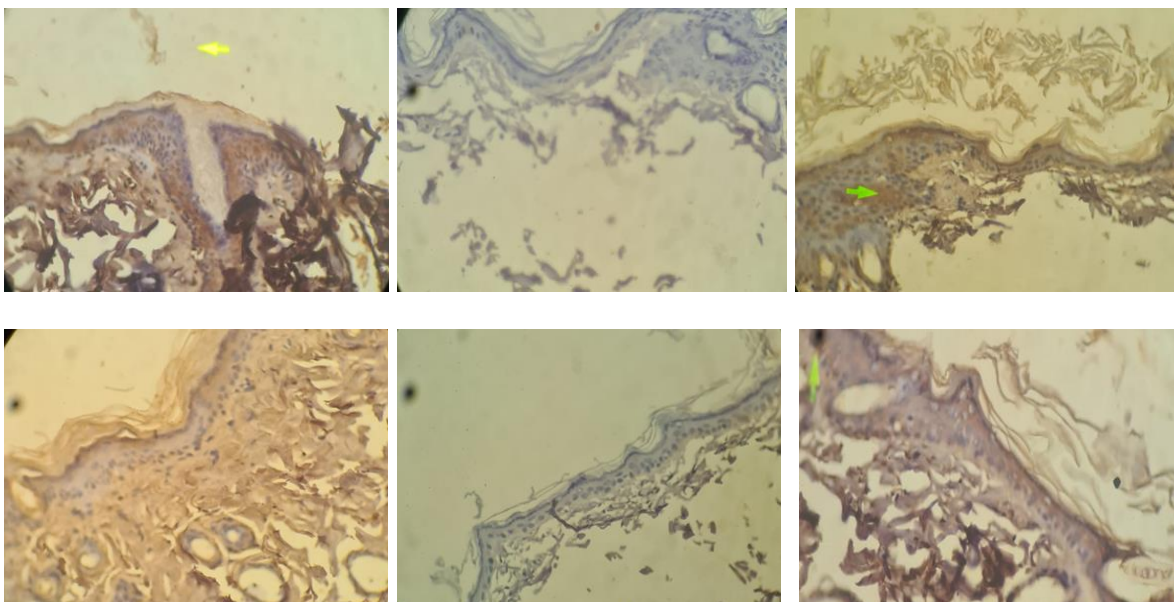
**Table 6.** Mann Whitney Test Result for TNF- $\alpha$

Treatment		p
I	II	
Control	Hydrocortisone	0,008
	Aloe vera	0,007
	Ozonated AV 300	0,005
	Ozonated AV 600	0,005
	Ozonated AV 1200	0,005
Hydrocortisone	Aloe vera	0,007
	Ozonated AV 300	0,005

Treatment		p
I	II	
	Ozonated AV 600	0,007
	Ozonated AV 1200	0,005
Aloe vera	Ozonated AV 300	0,005
	Ozonated AV 600	0,005
	Ozonated AV 1200	0,027
Ozonated AV 300	Ozonated AV 600	0,935*
	Ozonated AV 1200	0,029
Ozonated AV 600	Ozonated AV 1200	0,007

Significant p < 0,05, \* not significant

The Mann Whitney test performed and the results of Ozonated AV 300 group and the Ozonated AV 600 were not significant, while the other groups were significant.



**Figure 4.** Histopathological appearance with immunohistochemistry staining (400x)

**Notes:** From the top left in a clockwise direction shows the histopathological picture of

groups K, P1, P2, P3, P4, P5

## DISCUSSION

This study proved that administration of ozonated aloe vera were able to improve the healing of radiation dermatitis in *Sprague Dawley* rats compared to the control group in terms of the neutrophil-lymphocytes ratio. There were significant differences based on the results of data analysis between groups.

This study also concluded that the administration of ozoned aloe vera had been improving the healing process of radiation dermatitis in *Sprague Dawley* rats proved by significant differences of TNF- $\alpha$  expression among groups.

There were significant differences between the control group and the group administered with ozonated aloe vera in which improvement of the healing response were observed ( $p$  value < 0.05). This study showed that the total expression of TNF- $\alpha$  and the neutrophil-lymphocytes ratio in the groups undergone topical therapy intervention were significantly different compared to control group.

Radiation dermatitis causes inflammatory response and oxidative stress reactions resulting in cell damage, proinflammatory cytokines release, cell cycle change, and DNA damage. Tumor necrosis factor Alpha (TNF- $\alpha$ ) releases in the early phase of inflammation.<sup>4-7</sup> TNF- $\alpha$  is a proinflammatory cytokine produced by activated macrophages as an initial response to inflammation. A significant increasing in TNF- $\alpha$  manifests in the form of heat, pain, swelling and redness. TNF- $\alpha$  prolongs the inflammatory process by activating pro-inflammatory cytokines and other mediators such as nitric oxide and reactive oxygen species (ROS) that continue inflammation process and tissue injury.<sup>14</sup>

Neutrophils migrate to the site of tissue damage via chemotaxis during inflammation.<sup>15</sup> Neutrophils are first line immune system roled as defence mechanisms against infection by phagocytize debris, as well as against ROS.<sup>16</sup> Neutrophils play important role in amplifying

inflammation in the early stages of healing process and moreover signaling the late stages of healing against inflammation. During wound healing process , neutrophils undergo apoptosis after phagocytize the debris at the wound site and provide signals for resolution of inflammation. Continued recruitment of neutrophils or the accumulation of apoptotic neutrophils that are not regulated by macrophages can prolong inflammation and contribute to the development of chronic inflammation.<sup>10</sup>

The previous study concluded that ozone can reduce inflammation in dermatitis. The application of topical ozone in atopic dermatitis lesions was able to significantly reduce the number of inflammatory cells in the epidermal layer of papular lesions and edema in atopic dermatitis.<sup>17</sup> In addition, according to Robson P. et al<sup>18</sup>, ozone therapy on the oral mucosa of rats showed a significant difference in reducing the number of inflammatory cell infiltrates in the form of neutrophils and fibroblasts. Ozone can help improve skin's defence function, reduce inflammatory reactions, and accelerate wound healing. This healing results from ozone immunoregulatory mechanism that improves local perfusion and oxygen diffusion, antioxidant defence and epigenetic modification.<sup>11,17,19</sup> Increased oxygen perfusion at the wound site can increase the formation of granulation tissue resulting in increased speed of wound closure.<sup>20</sup>

The effect of ozone in this study was examined by observing the amount of TNF- $\alpha$  and neutrophil-lymphocyte ratio in adult male *Sprague Dawley* rats given radiation. The healing phase of the inflammatory process is usually characterized by decreased TNF- $\alpha$  activity<sup>14</sup> and decreased recruitment of neutrophils to tissues. Inflammatory mediators from neutrophils cause tissue damage, whereas lymphocytes have a modulating effect on neutrophils. High neutrophil-lymphocyte ratio indicates tissue damage as a result of higher neutrophil activity. Neutrophil-lymphocyte ratio is a marker to evaluate excitatory and inhibitory activity of the immune system in the healing phase of inflammation.<sup>21</sup>

The differences between negative control group compared to the group that was given aloe vera at doses of 300 mg/ml and 600 mg/ml were significant. The role of ozone as

immunoregulation and antioxidant defence against proinflammatory cytokines takes place effectively. Antioxidant systems are activated and antioxidant enzymes produced as a defence against oxidation and ROS (Reactive Oxygen Species). ROS cause various immunocytic and non-lymphoid cells involved in the wound repair process. ROS plays important role in the recruitment of lymphoid cells to the wound site for effective tissue repair. On the contrary, 1200 mg/ml ozonated aloe vera indicated overdose so that the anti-inflammatory effect was not effective.

The results of this study are in line with previous research conducted by Vahlepi et al.<sup>10</sup> administration of ozonated aloe vera to open wounds of *Sprague Dawley* rats showed significant wound healing and decreased neutrophil count compared to the control group that was not given ozonated aloe vera. A high neutrophil count in the wound area indicates that inflammation is still active. Neutrophils produce TNF- $\alpha$  and IL-6 that leads to continual inflammation.<sup>22</sup> The decrease number of neutrophils in wound site indicates an inhibition of the body's immune system to reduce the inflammatory reaction and that wound healing process has begun. The decrease number of neutrophils impacts the amount of TNF- $\alpha$  around the wound site which also reduce the inflammatory process so that wound healing occurs.

This study sum up that the lowest level of inflammation was in the group that received 2.5% hydrocortisone, followed by the group that was given ozonated aloe vera at a dose of 600 mg/ml, 300 mg/ml, aloe vera 0, aloe vera zoned at a dose of 1200. mg/ml, and the control group. The hydrocortisone group was the positive control group. In this study, the group that was given ozonated aloe vera at a dose of 1200 mg/ml had a lower inflammation level of 600 mg/ml, 300 mg/ml. The higher doses of aloe vera could not reduce the level of inflammation. The therapeutic index is the range of doses within which the drug is effective without unacceptable side effects. In animal studies, the therapeutic index can be calculated as the lethal dose of the drug for 50% of the population (LD 50) divided by the minimum effective dose for 50% of the population (ED 50). In clinical practice, the therapeutic index is the range of



doses over which a drug appears to be effective in clinical trials for a median of participants without unacceptable side effects. The therapeutic window is the range of drug concentrations within which the drug can exert an effect. The therapeutic window calculated by comparing the minimum toxic concentration with the minimum effective concentration. The minimum toxic concentration is the minimum concentration at which toxicity normally occurs. The minimum effective concentration is the minimum concentration required for a drug effect. Dosage has the effect of reducing the number of neutrophil lymphocyte ratio and TNF- $\alpha$  expression. At a dose of 1200 it was not able to reduce the number of neutrophil lymphocyte ratio and TNF- $\alpha$  expression like other lower doses because the dose had exceeded the therapeutic index and the therapeutic window.<sup>23</sup>

## **CONCLUSION**

1. Ozonated Aloe vera application was able to increase the healing process of radiation dermatitis in *Sprague Dawley* rats suffered from radiation dermatitis by decreasing the amount of TNF- $\alpha$  expression and NLR histopathologically.
2. Ozonated Aloe vera significantly decreased TNF- $\alpha$  expression proved by the group given ozonated aloe vera that had lower TNF- $\alpha$  expression compared to the control group on the 7th day after radiation administration.
3. Ozonated Aloe vera significantly reduced neutrophil-lymphocytes ratio based on the group that was given ozonated aloe vera that had lower NLR than the control group on the 7th day after radiation administration.

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1700) in coastal area of mohammedia. In total 40 samples of blue mussels (n=50 mussels) were collected from three points of at the mouth of the Oued El Maleh were analyzed for the detection of HEV and NoV using RT-PCR in real time. Overall, one (2%) of these samples tested positive for HEV RNA and thirteen (27%) for NoV. To our knowledge, this is the first notification of the detection of HEV and NoV in mussels collected in the mouth of Oued El Maleh. These findings suggest that a health risk may exist for users of waters in the the coast of Mohammedia and to consumers of shellfish. Monitoring HEV and NoV and similar viruses in shellfish can help prevent viral contamination. Further research is needed to assess the sources and infectivity of HEV in these settings, and to evaluate additional shellfish harvesting areas.

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# TOPICAL OZONATED ALOE VERA REDUCE THE INFLAMMATION IN RADIATION DERMATITIS

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## Keywords:

radiation dermatitis, ozonated aloe vera, TNF- $\alpha$  expression

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## ABSTRACT

Radiation dermatitis is a side effect is the most common side effect of external radiation with varied clinical features. Applying topical ozonated aloe vera can reduce the inflammatory reaction in radiation dermatitis by decreasing neutrophil migration and TNF- $\alpha$  expression. This study aims to improve the healing response of radiation dermatitis with ozonated aloe vera therapy based on TNF- $\alpha$  face and the ratio of neutrophils to lymphocytes. Experimental study with post-test only randomized control group design on 36 male *Sprague Dawley* rats weighing 150 $\pm$ 50 grams and given a single dose of 7 Gy radiation. After radiation, acclimatization was carried out for seven days and followed by randomization and divided into six groups, namely negative control, positive control given hydrocortisone cream 2.5%, treatment groups 1, 2, 3, and 4 were given aloe vera, aloe vera, respectively ozonated doses of 300 mg/mL, 600 mg/ml, and 1200 mg/mL for seven days. After the treatment, the rats were terminated, and histological preparations were made with HE staining and immunohistochemistry to see the NL ratio and TNF- $\alpha$ . Three rats were dead on external radiation administration. In the Kruskal-Wallis test, there were significant differences in the NL ratio and TNF- $\alpha$  expression based on the type of treatment ( $p < 0.05$ ). In the Mann-Whitney test, there were significant differences between treatment groups ( $p < 0.05$ ). Ozonated aloe vera increases radiation dermatitis's healing response in Sprague Dawley rats by decreasing TNF- $\alpha$  expression and neutrophil-lymphocyte ratio.



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## 1. INTRODUCTION

Radiation dermatitis is the most common side effect and presented in up to 95% of patients after external radiation administration [1]. The clinical feature of radiation dermatitis varies from skin reactions such as mild erythema and desquamation to skin necrosis. In chronic conditions, lesions appear in the form of skin atrophy and telangiectasis [2- 4]. Incidence and severity vary depending on extrinsic factors such as the type of radiation such as local and locoregional, radiation dose, and the area of radiation administration [5], [6].

Skin is an organ that is constantly dividing. The layer that contains keratinocytes and melanocytes is radiosensitive. Hence radiation exposure can affect the cell division cycle. Repeated radiation exposure led to decreased time for cell repair and increased cell injury [2], [3]. Cell damage after radiotherapy causes changes in the cell cycle, cytokine production, and DNA damage. In the early phase of inflammation, proinflammatory cytokines appear, such as IL-1, IL-3, IL-5, IL-6, and Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ), which cause infiltration eosinophils and neutrophils so that the tissue damage is getting worse. Excessive inflammatory cell response can interfere with wound healing due to disruption of granulation tissue formation, fibrogenesis, and angiogenesis [1], [4], [6], [7].

Aloe vera is widely used as a topical therapy for skin tissue inflammation due to its anti-inflammatory and antioxidant properties. The mechanism of cyclooxygenase inhibition and pro-inflammatory cytokines, thereby reducing neutrophil infiltration and the ability to reduce platelet aggregation, leads to repairment of tissue inflammation and acceleration of wound healing [8]. Ozonated aloe vera has a remarkable effect because it can provide oxygen and regulate antioxidant enzymes to protect against oxidative reactions and increase healing factors [9- 12]. Administration of topical ozonated aloe vera aid the regulation of wound healing without causing toxic effects because the ozone acts immediately with fat in the stratum corneum layer of the skin and is absorbed as an antioxidant through the skin, [9]

Topical ozonated aloe vera provides significant benefits in cancer patients with radiation dermatitis [13]. Therefore, the authors tried to elaborate an increased healing response of radiation dermatitis based on TNF- $\alpha$  expression and the neutrophils lymphocytes ratio in Sprague Dawley rats treated with ozonated aloe vera therapy compared to no treatment and 25% hydrocortisone cream.

## 2. METHODS

This was an experimental study with a post-test-only control group design. The 36 male Sprague Dawley rats from the Experimental Animal Laboratory, Sultan Agung University, Semarang, aged 2-3 months, weighing  $150\pm 50$  grams, were acclimatized for a week. Rats with physical abnormalities appearing inactive and dead were excluded from the study. The rats were anesthetized intraperitoneally using a mixture of ketamine 80 mg/kg BW and xylazine 10 mg/kg BW. They received 7 Gray electron beam radiotherapy from a linear accelerator (Siemens Primus<sup>TM</sup>) on the 4x4 cm area of the back of the rats. Ozonated aloe vera was obtained from the Plasma Research Laboratory of Diponegoro University. The rats were randomly divided into six groups consisting of 6 rats in each group and treated with:

- i. C1 (Negative control): radiation without therapy
- ii. C2 (Positive control): radiation + topical hydrocortisone 2,5%
- iii. T1; radiation + topical aloe vera
- iv. T2; Radiation + 300 mg/mL of ozonated aloe vera
- v. T3; radiation + 600 mg/mL of ozonated aloe vera
- vi. T4; radiation + 1200 mg/mL of ozonated aloe vera

After seven days of treatment, the rats were then terminated. The wound tissue was incised; then histological preparations were made. The staining was performed using hematoxylin-eosin (HE) to see the ratio of neutrophils and lymphocytes and immunohistochemical staining to see the expression of TNF- $\alpha$  (Figure 1). The assessment of TNF- $\alpha$  was conducted using the Allred score (Table 1). The Faculty of Medicine Universitas Diponegoro Health Research Ethics Committee approved this study (No. 70/EC/H/FK-UNDIP/VII/2021).

Data analysis includes descriptive analysis and hypothesis testing. In the descriptive analysis, the dependent variable is presented in the form of a table of mean, SD, median, and box plot graphs. The hypothesis was tested using the Kruskal Wallis Non-Parametric test, followed by the Mann-Whitney test to determine the differences between groups. It was considered significant if  $p < 0.05$  with a 95% confidence interval.

### 3. RESULTS

Three of the 36 rats given radiation died, leaving 33 rats. The rats were then randomly divided into six groups, namely 5 in the NCG, PCG, and T1 groups and 6 in the T2, T3, and T4 groups. After seven days of treatment, termination was carried out and continued with histopathological examination for the total neutrophil-lymphocyte ratio and TNF $\alpha$  expression. In HE staining, the lowest number of NL ratios was found in the PCG group ( $1.15 \pm 0.77$ ), followed by the treatment group, which was given ozonated aloe vera at a dose of 600 mg/ml, 300 mg/ml, 1200 mg/ml, aloe vera and the lowest ( $0.02 \pm 0.04$ ) in PCG (Figure 2).

In the Kruskal Wallis test, there was a significant difference in the NL ratio based on the type of treatment with  $p < 0.05$ . The Mann-Whitney test was performed to determine the differences between the treatment groups. Based on the Mann-Whitney test, there were significant differences between the treatment and control groups, except in the PCG and T2 groups, where the differences were not significant ( $p = 0.072$ ,  $p < 0.05$ ) (Table 2).

In histopathological examination with immunohistochemical staining, TNF- $\alpha$  expression was measured using the Allred score, and the result obtained was a decrease in TNF- $\alpha$  expression (Figure 3). The results of statistical analysis with the Kruskal Wallis test obtained a  $p$ -value  $< 0.05$ , so it can be concluded that there is a significant difference in TNF- $\alpha$  based on the type of treatment, with the highest TNF- $\alpha$  average ( $6.76 \pm 0.41$ ) in NCG and the lowest TNF- $\alpha$  average ( $3.72 \pm 1.13$ ) in the PCG group (Figure 4). Mann Whitney test was performed to determine differences between treatment groups. From the results of the Mann Whitney test, the difference between the P2 and P3 groups was not significant ( $P = 0.935$ ,  $p < 0.05$ ), while between other treatment groups, significant differences were obtained (Table 2).

### 4. DISCUSSION

Radiation dermatitis occurs due to the inflammatory response and oxidative stress reactions that cause cell damage, causing changes in proinflammatory cytokines, cell cycle changes, and DNA damage. One of the proinflammatory cytokines that appear in the early phase of inflammation is TNF- $\alpha$  due to macrophage activation [1], [4], [6], [7]. A significant increase in TNF- $\alpha$  has indicated the cardinal signs of inflammation in heat, pain, swelling, and redness. TNF- $\alpha$  enhances and prolongs the inflammatory process by activating pro-inflammatory cytokines and other mediators such as nitric oxide and reactive oxygen species (ROS) that cause continued inflammation and tissue injury [14]. When inflammation occurs, neutrophils migrate to the site of tissue damage by chemotaxis. Neutrophils are recruited in large numbers to phagocytize debris. They are the body's first line of defense against infection, including ROS [15]. Neutrophils play a role in amplifying inflammation in the early stages of healing and providing signals in the late stages of healing against inflammation. Continued recruitment of neutrophils or the accumulation of apoptotic neutrophils that are

unregulated by macrophages can prolong inflammation and contribute to the development of chronic inflammation [10].

The effect of ozone in this study was reviewed by looking at the amount of TNF- and the NL ratio in adult male Sprague Dawley with radiation dermatitis. The healing phase of the inflammatory process is usually marked by a decrease in the Allred score, [16], and a reduction in the recruitment of neutrophils to tissues. A high NL ratio can indicate tissue damage and can be used to evaluate the excitatory and inhibitory activity of the immune system in the healing phase of inflammation [17].

Based on previous research, ozone content can reduce inflammation in dermatitis. The use of topical ozone in atopic dermatitis lesions significantly reduced the number of inflammatory cells in the epidermal layer of papular lesions and edema in atopic dermatitis [18]. The results of data analysis between the negative control group compared the group given ozonated aloe vera at doses of 300 mg/ml and 600 mg/ml found a significant difference. Meanwhile, there was an excess dose in the administration of ozonated aloe vera at 1200 mg/ml, so the anti-inflammatory effect was ineffective. The results of this study are in line with the research of [10] where the administration of ozonated aloe vera on open wounds of Sprague Dawley rats showed wound healing in the form of a decrease in the number of neutrophils compared to the negative control group.

Ozone influences wound healing, a multiphase process consisting of four phases: hemostasis, inflammation, proliferation, and remodeling [19]. This effect occurs because of an immunoregulatory mechanism, which improves local perfusion and oxygen diffusion, antioxidant defense, and epigenetic modification [11], [18]. Increased oxygen perfusion at the wound site can increase granulation tissue formation, increasing the speed of wound closure [20]. A high neutrophil count in the area around the wound indicates that inflammation is still active. Neutrophils will produce TNF- $\alpha$  and IL-6, pro-inflammatory cytokines, so inflammation will continue to occur [21]. A decrease in the number of neutrophils in the area around the wound indicates an inhibition of the body's immune system to reduce the inflammatory reaction and suggests that wound healing has started. The decrease in neutrophils will impact the amount of TNF- $\alpha$  around the wound, reducing the inflammatory process, so wound healing occurs.

Our study showed that ozonated aloe vera exhibit a wound-healing effect on radiation dermatitis by reducing TNF- $\alpha$  expression and NLR ratio following a single radiation exposure on the skin. However, our study was only conducted with single radiation exposure and measured on a single-time event. Further study regarding the long-term effect on a more extended evaluation period would be beneficial.

## **5. CONCLUSION**

Ozonated aloe vera increases radiation dermatitis's healing response in Sprague Dawley rats by decreasing TNF- $\alpha$  expression and neutrophil-lymphocyte ratio.

## **DECLARATION**

Ethics approval

Ethics was obtained from the Ethical Commission for Health Research, Faculty of Medicine, Diponegoro University with the Ethical Clearance number No. 70 / EC / H / FK.UNDIP / VII / 2021. All experimental animals had been cared and managed accordingly to animal maintenance standards

Consent for publication

Not applicable

#### Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

#### Competing interests

All authors report no competing interest

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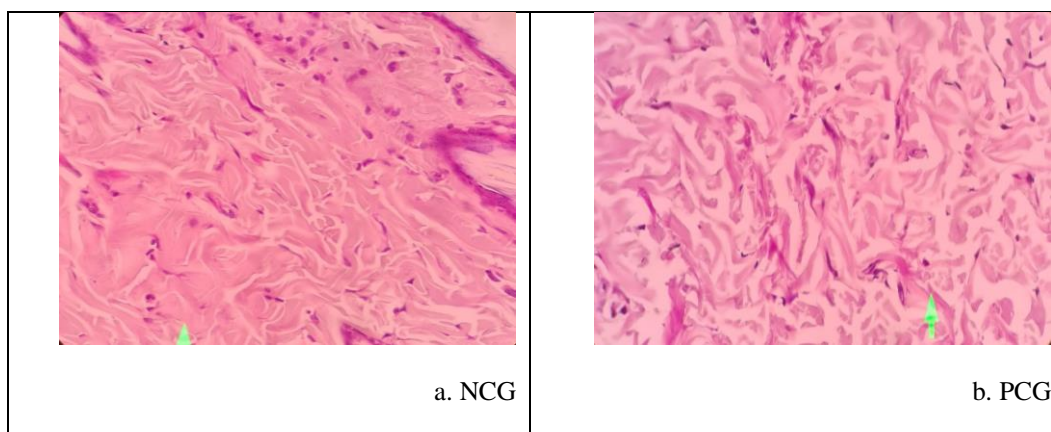
**Table 1.** Assessment of TNF- $\alpha$  using the Allred score [24]

Stained cell (%)	Proportion Score (A)	Intensity	Intensity Score (B)	Final Score (A+B)
0	0	None	0	0
<1	1	Weak	1	1-2
1 - 10	2	Medium	2	3-4
11 - 33	3	Strong	3	4-6
34 - 66	4	-	-	4-7
>67	5	-	-	5-8

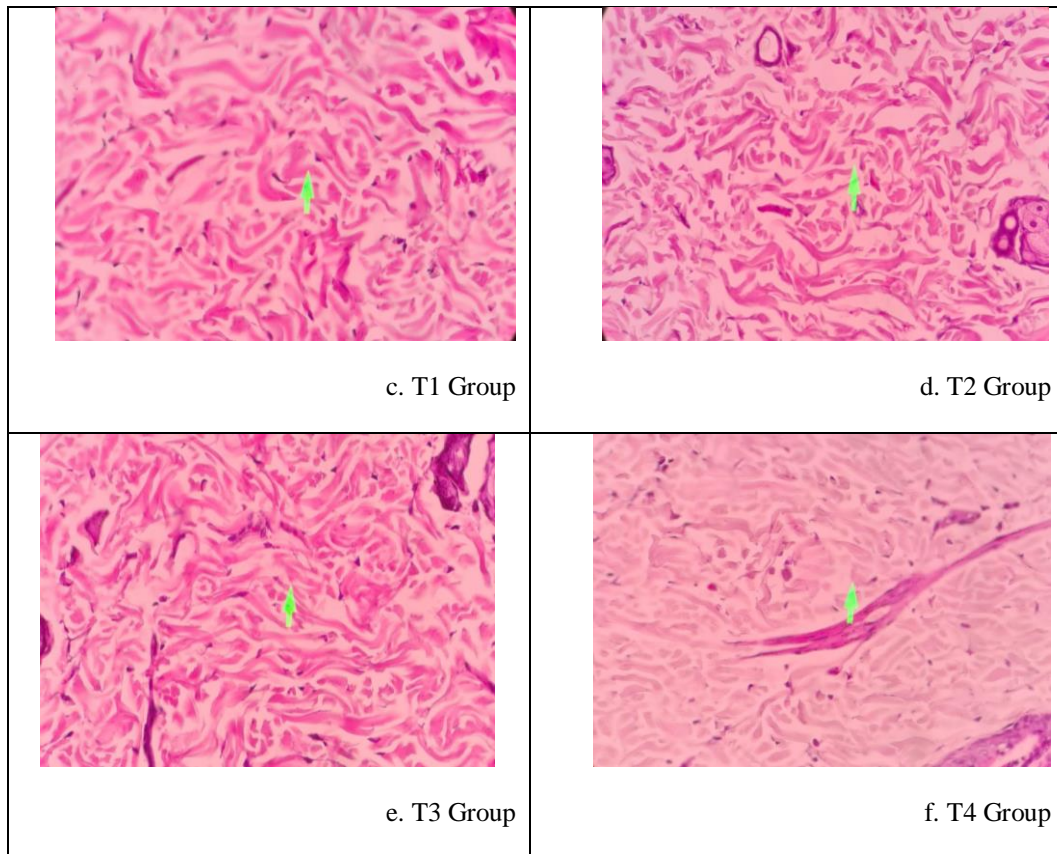
**Table 2.** Mann Whitney test results of the NL ratio and TNF- $\alpha$ .

Treatment Type		P	P
I	II	(NL Ratio)	(TNF- $\alpha$ Expression)
NCG	PCG	0,007*	0,007*
	T1	0,012*	0,012*
	T2	0,006*	0,008*
	T3	0,006*	0,007*
	T4	0,009*	0,005*
PCG	T1	0,007*	0,005*
	T2	0,005*	0,005*
	T3	0,072	0,007*
	T4	0,007*	0,005*
T1	T2	0,006*	0,005*
	T3	0,006*	0,005*
	T4	0,015*	0,027*
T2	T3	0,007*	0,935
	T4	0,005*	0,029*
T3	T4	0,006*	0,007*

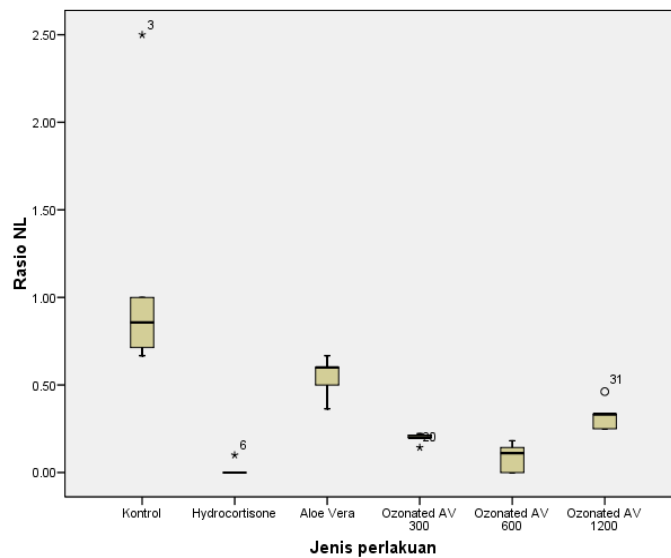
Significant  $p < 0,05$ , \*significant



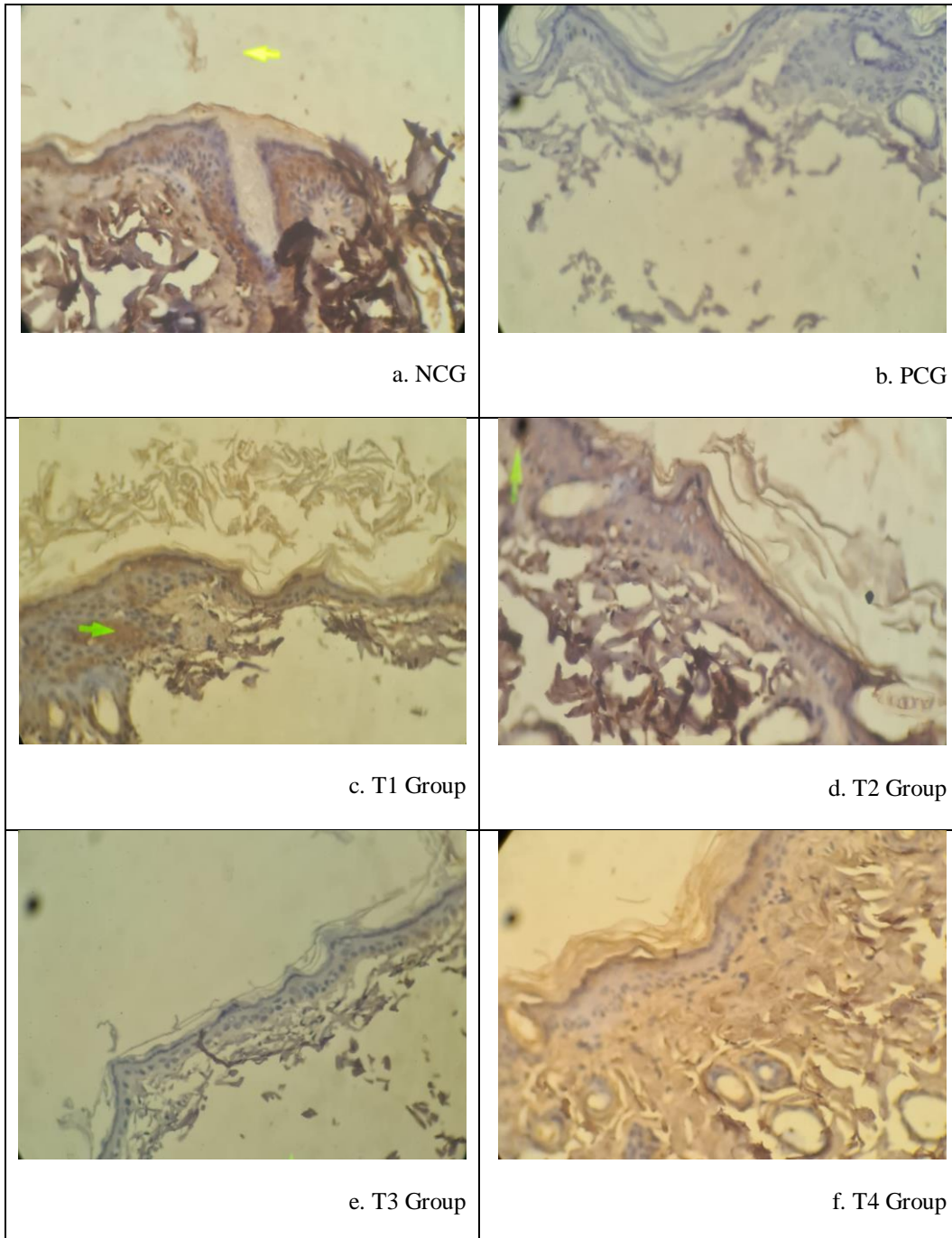




**Figure 1.** Histopathological appearance of neutrophils (green arrows) with HE staining at 400x magnification.

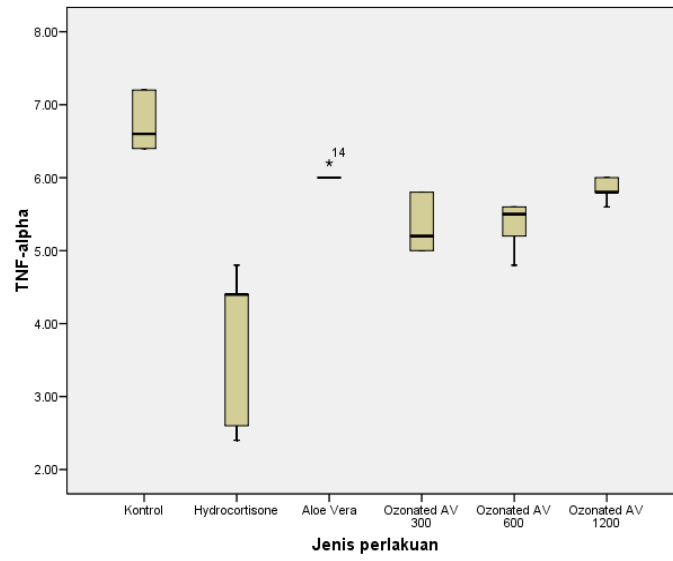


**Figure 2.** NL ratio on box plot graphic.



**Figure 3.** Histopathological appearance of TNF- $\alpha$  expression by immunohistochemical staining at 400x magnification.





**Figure 4.** TNF- $\alpha$  expression on boxplot graphic.