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# The Effect of Multilevel Doses of Caffeine on Tissue Macrophage and Blood Lymphocyte Count in Autologous Full Thickness Skin Graft Healing in Sprague Dawley Rats

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### ABSTRACT

**Backgrounds.** A skin graft is one of the routine surgical procedures performed. This procedure gives excellent results when done as early as possible after the trauma. Caffeine has a mechanism as an adenosine-receptor A2 antagonist which can induce wound healing through increased angiogenesis. This study aimed to determine the efficacy of coffee caffeine in the initiation of full thickness skin graft autologous wound healing by assessing the number of macrophages and lymphocytes in Sprague-Dawley rats. **Methods:** The research design is an experimental study with a post-test-only approach with a control group design. Twenty male Sprague Dawley rats aged 15 weeks (140 – 150 grams) were randomly divided into four groups. One group became the control group (decaffeinated) while the other group received various doses of caffeine (3 mg/kgBW, 6 mg/kgBW, and 9 mg/kgBW). Autologous skin grafts were performed in all groups. HE examination was performed to confirm the number of macrophage cells in the tissue. Data analysis was carried out with the help of SPSS 25 software. **Results:** There were significant differences in the number of tissue macrophages in the four groups. The group that received 9 mg/kgBW of caffeine showed the highest number of macrophages compared to the other groups. Based on the number of lymphocytes in the peripheral blood, the group that received a dose of 6 mg/kg BW showed the highest number of lymphocytes compared to other groups. **Conclusion:** Caffeine showed the ability to initiate full thickness skin graft autologous wound healing by increasing the number of macrophages and lymphocytes in Sprague Dawley rats.

### 1. Introduction

Wound healing is one of the most complex processes in human physiology. This process involves a series of reactions and interactions between cells and mediators which are divided into several phases. The inflammatory phase aims to remove dead tissue and prevent infection. The proliferative phase is characterized by the formation of granulation tissue accompanied by a rich network of new blood vessels, fibroblasts, and macrophages in a loose supporting tissue. The second phase which takes place from the

8<sup>th</sup> to the 21<sup>st</sup> post-injury day is a phase of epithelialization and at the same time provides a reflection in wound care to be able to achieve a wound condition that has been covered with epithelium. The last phase is the maturation phase which is characterized by a balance between the formation and degradation of collagen. There are at least 3 prerequisites for local conditions so that the wound healing process can take place normally, namely, all tissue in the wound area and its surroundings must be

vital, free of foreign objects, and not accompanied by excessive contamination or infection.<sup>1-4</sup>

Various choices of various therapeutic models in wound healing, a skin graft is one of the therapies of choice in the rapidly growing wound healing process. Skin grafts have been carried out in India since 2000 years ago but did not develop until the 19<sup>th</sup> century. In the early 19<sup>th</sup> century, skin grafts were introduced in the western world. Over the past 100 years, the tools and methods used have undergone many changes.<sup>4-6</sup> As of 2012, more than 410,000 burns occurred in the United States, with approximately 40,000 requiring hospitalization. Research reveals that in India more than 1 million people suffer burns every year. Data compiled by the Ministry of Health in 2015, showed that burn trauma was the 6<sup>th</sup> for unintentional injury with a total of 7.7%.<sup>2</sup> Data in Indonesia, burns reach 195,000 deaths every year and rank 9<sup>th</sup> overall for people aged 5-14 years with an estimated 41,575 deaths. Ranked 15<sup>th</sup> in the age range 0-4 years and 15-29 years with deaths reaching 62,655 deaths and 47,067 deaths. This makes burns the 7<sup>th</sup> injury in the world, the number of deaths is 5% of all burns.<sup>2</sup>

A skin graft is one of the routine surgical procedures performed. This procedure gives excellent results when done as early as possible after the trauma.<sup>7-9</sup> Wounds that cannot be closed primarily can be closed in various ways, including skin grafts. The success of the skin graft is also determined by the preoperative and postoperative care of the skin graft.<sup>7</sup> Skin grafts require sufficient vascularity to survive before and after a close relationship with the recipient tissue. After the skin is removed from the donor, the skin turns pale because it is cut off from the blood supply where the capillaries in the graft contract and the red blood cells are squeezed out. After the graft is attached to the recipient, a gradual change in the color of the graft to pink as if there is re-circulation occurs due to the passive movement of free red blood cells into the graft capillaries. The capillary effect occurs during the first 12 hours. Nutrition in the skin graft begins with the process of plasmatic circulation where there is a process of imbibition of plasma/serum and oxygen into the graft.<sup>7-9</sup>

The immune system has an important role in the healing of skin graft wounds. The activity of macrophages as chemoattractants plays a role in summoning pro-inflammatory cytokines such as TNF- $\alpha$ , IL-6, IL-10 to cause a healing cascade by promoting the stimulation of VEGF (vascular endothelial growth factor) and ensuring the supply of fibroblasts can achieve the goal to create collagen for donor attachment. Macrophages and lymphocytes as cells that play a role in the body's defense system are very helpful in creating an infection-free wound healing atmosphere where these conditions are indispensable for wound healing.<sup>10,11</sup>

Coffee contains caffeine (1,3,7-trimethylxanthine) as an antioxidant which is often said to have an important role in wound healing. Previous studies have revealed that caffeine has a mechanism as an adenosine-receptor A2 antagonist which can induce wound healing through increased angiogenesis. It was also revealed in another study that it was found that caffeine can inhibit wound healing by inhibiting epithelialization and suppressing cell proliferation through its anti-inflammatory effect where inflammatory mediators are needed during wound healing to stimulate various vascular endothelial growth factors.<sup>12,13</sup> This study aimed to determine the efficacy of coffee caffeine in the initiation of full thickness skin graft autologous wound healing by assessing the number of macrophages and lymphocytes in Sprague Dawley rats.

## **2. Methods**

The research design is an experimental study with a post-test-only approach with a control group design. The research was conducted at the experimental animal laboratory of the Faculty of Medicine, Diponegoro University, Semarang, the Anatomical Pathology Laboratory, Clinical Pathology, Dr. Kariadi, and Diponegoro National Hospital Semarang with a research time of April – June 2018. This research has also received Ethical Clearance with No.69/EC/H/DKRSKD/VI/2018.

Twenty male Sprague Dawley rats aged 15 weeks (140 – 150 grams) were adapted in the laboratory for

one week. After going through the selection process and being declared eligible, the twenty rats were randomly divided into four groups equally. Autologous skin grafts were performed in all groups. All rats were treated equally postoperatively and had the same diet and drinking ad libitum. One group became a control group receiving no caffeine diet, while another group according to the group received different doses of *caffeine* (Caffeine anhydrous, ® Sigma-Aldrich) in each group orally every day. Group K as the control group was not given any treatment. Group P1 as treatment 1 received a low dose of caffeine per oral 3 mg/kg once per day, group P2 as treatment 2 received a moderate dose of caffeine per oral 6 mg/kg once per day, group P3 as treatment 3 received a high dose of caffeine per day. orally 9 mg/times per day. Caffeine was administered on the first day after autologous skin graft treatment, then observed for 1 week. The next process, 1 week later, was anesthetized using ketamine-xylazine for tissue harvesting that had been done with autologous skin grafts and peripheral blood sampling in rat tails followed by termination through cervical

vertebral dislocation in anesthetized rats. All skin graft and autologous tissues were then processed into paraffin block preparations. Each preparation was cut to a thickness of 4 microns and HE examination was performed to confirm the number of macrophage cells in the tissue. Peripheral blood preparations were made with Giemsa staining slides to confirm the number of lymphocytes in the peripheral blood.

A normality test with Saphiro Wilk was carried out to assess the normality of the data. Data were analyzed by the Kruskal-Wallis test and post hoc Mann-Whitney Test. The difference was declared significant if the P-value <0.05 was obtained with a 95% confidence interval. Data analysis was performed with the help of SPSS 25 software

### 3. Results

Table 1 shows that there were significant differences in the number of tissue macrophages in the four groups. The P3 group showed the highest number of macrophages compared to the other groups.

Table 1. Description of the number of macrophages between groups

Group	Median (min-max)	p*
K	4 (3 – 5)	0.001
P1	4 (4 – 5)	
P2	7 (5 – 8)	
P3	14 (12 – 14)	

\* Kruskal Wallis Test (significant p < 0.05)

Table 2. Post Hoc analysis of macrophage numbers between groups

Group	P1	P2	P3
K	0.343	0.011*	0.008*
P1	–	0.009*	0.006*
P2	–	–	0.008*

\* Mann Whitney Test (significant p < 0.05)

Table 2 shows a significant difference between groups K and P2 (p = 0.011), K and P3 (p = 0.008); P1 to P2 (p = 0.009); P1 to P3 (p = 0.006); and P2 with P3

(p = 0.008). A non-significant difference was found between groups K and P1 (p = 0.343).

Table 3. Description of lymphocyte number between groups

Group	Median (min-max)	p
K	12 (7 – 21)	0.006
P1	12 (4 – 28)	
P2	14 (0 – 34)	
P3	11 (3 – 12)	

\* Kruskal Wallis Test (significant  $p < 0.05$ )

Table 3 shows that there are significant differences in the number of peripheral blood lymphocytes in the

four groups. The P2 group showed the highest number of lymphocytes compared to the other groups.

Table 4. Post Hoc analysis of the number of lymphocytes between groups

Group	P1	P2	P3
K	0.306	0.008*	0.009*
P1	–	0.008*	0.008*
P2	–	–	0.032*

\*Mann Whitney Test (significant  $p < 0.05$ )

Table 4 shows significant differences between groups K with P2 ( $p = 0.008$ ), K with P3 ( $p = 0.009$ ); P1 to P2 ( $p = 0.008$ ); P1 to P3 ( $p = 0.008$ ); and P2 with P3 ( $p = 0.032$ ). A non-significant difference was found between groups K and P1 ( $p = 0.306$ ).

#### 4. Discussion

Caffeine has a mechanism as an adenosine-receptor(A2) antagonist which can induce wound healing by increasing the number of macrophages and resulting in a synergistic upregulation of vascular endothelial growth factor (VEGF).<sup>14</sup> Another study stated that there was a close relationship with the effect of increasing macrophages with the addition of dietary caffeine in the process of accelerating wound healing.<sup>7</sup> During the skin graft wound healing process, the immune system has an important role. The activity of macrophages as chemoattractants plays a role in summoning proinflammatory cytokines such as TNF- $\alpha$ , IL-6, IL-10 to cause a healing cascade by promoting the stimulation of VEGF (Vascular endothelial growth factor) and ensuring the supply of fibroblasts can achieve the goal to create collagen for donor attachment. Macrophages and lymphocytes and monocytes as cells that play a role in the body's defense

system are very helpful in creating an infection-free wound healing atmosphere where these conditions are indispensable for wound healing.<sup>15,16</sup> One of the important functions of macrophages is their ability to promote angiogenesis through the initiation of VEGF production. VEGF is a potential pro-angiogenic factor wherein several studies have shown VEGF has a 50% contribution to angiogenic activity in wounds.<sup>17,18</sup>

Caffeine has the effect of modulating the number of inflammatory cells.<sup>19</sup> The improvement, in this case, is that the number of lymphocytes increased in the group receiving low to moderate doses of caffeine, but there was a decrease in the number of lymphocytes in the high dose group. This finding is consistent with a study that reported that dietary caffeine in a rat model could reduce the number of proinflammatory cytokines.<sup>20</sup> As previously explained that caffeine and adenosine are interrelated in the wound healing process through the immune system. Proinflammatory cytokines consist of IL-1, IL-6, and TNF- $\alpha$ , where are the main elements involved in the inflammatory stage of the wound healing process. IL-1 is produced immediately upon tissue damage by macrophages and lymphocytes while TNF- $\alpha$  stimulates fibroblast production and FGF release. The release of IL-6 and TNF- $\alpha$  from activated

lymphocytes and macrophages that infiltrate the site of tissue damage is a very essential process to initiate the wound healing process and promote reepithelialization.<sup>21,22</sup> IL-6 remains present during the inflammatory stage of wound healing, it stimulates proliferative and mitogenic activity in keratin cells and attracts lymphocytes to come to the site of tissue damage. TNF- $\alpha$  indirectly increases reepithelialization by stimulating VEGF production.<sup>23-25</sup>

## 5. Conclusion

Caffeine showed the ability to initiate autologous skin graft full thickness wound healing by increasing the number of macrophage cells and lymphocytes in the Sprague Dawley rats.

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