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## Effects of 5% astaxanthin gel on angiogenesis and granulation tissue in second-degree burn animal model

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### Abstract

**Background:** Burn injuries generate more free radicals and lead to complex immune dysfunction, which can delay wound healing compared to other wound types. Angiogenesis, essential for wound healing, requires granulation tissue as a marker for successful wound healing. Low concentrations of reactive oxygen species (ROS) are necessary to initiate this process. Astaxanthin is a potent antioxidant with anti-inflammatory properties, known to activate angiogenesis and modulate ROS signaling during wound healing.

**Methods:** This experimental study aimed to evaluate 5% astaxanthin gel on second-degree burns using 30 male Wistar rats. Simple random sampling was utilized. A total of 6 groups were divided according to the time the lesions were evaluated i.e., I. Normal saline, day 2 (n=5); II. Normal saline, day 5 (n=5); III. Normal saline, day 7 (n=5); IV. 5% astaxanthin gel, day 2(n=5); V. 5% astaxanthin gel, day 5 (n=5); VI. 5% astaxanthin gel, day 7 (n=5). Histological assessment of angiogenesis and granulation tissue was based on the number of blood vessels and the extent of the wound to the dermis, respectively.

**Results:** The mean of angiogenesis in 5% astaxanthin gel group was higher than the control group on day 5 ( $p$ -value < 0.05). The positive correlation between angiogenesis and granulation tissue was observed on day 5 ( $p$ -value < 0.05).

**Conclusion:** The primary effect of 5% astaxanthin gel is during the proliferative phase of wound healing in second-degree burns.

**Keywords:** *angiogenesis, astaxanthin, granulation tissue, second-degree burn*

### Background

A burn is a type of wound that causes tissue damage or loss due to exposure to heat sources. It is estimated that 180,000 deaths occur each year due to burns, with a majority of cases occurring in developing and lower-middle-income countries.<sup>1</sup> A report on burn injuries from the Burn Unit of the Regional General Hospital Dr. Saiful Anwar Malang (RSSA) discloses approximately 177 burn cases with a mortality rate of 13% in 2018. In 2019, there was an increase to 181 cases with a mortality rate of 17% (unpublished data). The most common type of burn is scald burn typically caused by boiling water and direct heat contact. Most scald burns are classified as second-degree burns, which damage the entire epidermis and part of the dermis, leading to inflammatory responses and

exudation.<sup>2</sup> Burns possess distinctive characteristics and present challenges to physicians in assessing the burn's width and depth. This is due to increased free radicals, immune dysfunction involving more inflammatory processes, and a more complex microenvironment complicating the wound-healing process.<sup>3,4</sup> In burn injuries, the tissues are severely damaged, cells and blood vessels are often broken.<sup>5</sup> The angiogenesis process is needed in wound healing. It involves the growth of new capillaries to form granulation tissue. The capillary proliferation carries oxygen and micronutrients to the developing tissue.<sup>6</sup> Factors influencing angiogenesis include hypoxic conditions, inflammation, and growth factors. Current optimal wound healing strategies aim to generate growth factors to the wound bed, thereby increasing

angiogenesis and the formation of granulation tissue.<sup>7,8</sup>

Silver sulfadiazine (SSD) is currently the gold standard in topical burn treatment of second- and third-degree burns. Its efficacy in wound care is attributed to its antibacterial properties, but the microbial resistance to antibacterial compounds is becoming increasingly concerning.<sup>9</sup> Another strategy to enhance burn wound healing involves controlling the amounts of free radicals during the wound healing process by administering antioxidants.<sup>7</sup> The best-known antioxidants are vitamins C and E, which produce new free radicals which are weaker when metabolized.<sup>8</sup> Presently, astaxanthin is recognized for its superior efficacy compared to other antioxidants, as it can remain both inside and outside the cell membrane. The structure of astaxanthin's conjugated double bonds acts as a powerful antioxidant that can donate electrons and convert free radicals into more stable products, thereby terminating the free radical chain reaction.<sup>9,10</sup> Furthermore, astaxanthin also has anti-inflammatory effects and is present mainly in aquatic organisms in the ocean. As a maritime nation, Indonesia has begun to develop a microalgae-based biotechnology industry that produces astaxanthin products from the *Haematococcus pluvialis* strain.<sup>11</sup>

In wound healing, astaxanthin can activate the physiological process known as angiogenesis by regulating the levels of reactive oxygen species (ROS), ensuring they are not harmful to endothelial cells.<sup>12</sup> Both in vitro and in vivo studies have shown that astaxanthin can protect against cell or tissue damage caused by oxidative stress, maintaining physiological functions by regulating the cellular redox state. Topical astaxanthin concentrations range from 0.01% to 5%, with formulation at 5% demonstrating the highest antioxidant activity.<sup>13</sup> Topical astaxanthin has also been investigated in the context of photoaging.<sup>8,14</sup> However, to the best of our knowledge, no studies have reported on the use of topical astaxanthin for burn treatment to date. Thus, this study aims to examine the effect of 5% astaxanthin gel on angiogenesis and granulation tissue formation using an animal model with second-degree burns.

## Methods

### Animals

This is an experimental study with a post-test-only design using 30 *Rattus norvegicus* Wistar strain

rats. The inclusion criteria were healthy male Wistar rats, aged 3 months, weighing between 200-250 grams. The exclusion criteria were diseased male Wistar rats (exhibiting symptoms such as hair dullness/loss or baldness, inactive motion, abnormal discharge of exudate from the eyes, mouth, anal, or genitals), weight loss of more than 10% of initial weight in the acclimation phase or died during the treatment period.

The sampling method was simple random sampling consisting of 6 groups based on the time points when the lesions were evaluated, i.e., I. Normal saline group, biopsy taken on day 2 (n=5); II. Normal saline group, biopsy taken on day 5 (n=5); III. Normal saline group, biopsy taken on day 7 (n=5); IV. 5% astaxanthin gel group, biopsy taken on day 2 (n=5); V. 5% astaxanthin gel group, biopsy taken on day 5 (n=5); VI. 5% astaxanthin gel group, biopsy taken on day 7 (n=5).

Skin tissue samples were taken on days 2, 5, and 7 post-induction of second-degree scald burn to observe angiogenesis (number of blood vessels) and granulation tissue (surface of the wound to the dermis). The study was approved by the Animal Care and Use Committee, Number 056-KEP-UB-2020. The experimental animals were acclimated for 7 days at the Biomedics Laboratory Universitas Brawijaya, Malang, Indonesia. All were fed pellets and water ad libitum. The experimental rats were housed in individual cages with adequate ventilation in a clean area. After the study, all experimental animals were euthanized by cervical dislocation under anaesthesia.

### Scald burn induction

Ketamine anaesthesia (60 mg/kg) was administered via intramuscular injection to all experimental animals. Then, the backs of the rats were shaved according to the predetermined area of burns. The burn was induced using a 2 cm diameter metal circle, heated to 100°C, and applied for 5 seconds to the rats' back skin.<sup>15</sup> After the burn was created, topical therapy was given according to the determined groups.

### Astaxanthin gel

The astaxanthin gel used was derived from *Haematococcus pluvialis* with a concentration of 5%, formulated using nanoemulsion technology with an IC50 of <3 ppm. Other compositions included were surfactants (Tween 80 and Cremophor RH40), co-surfactant (PEG 400), and a carbomer gelling agent.

## Wound care

The burned area was cleaned with normal saline in the group treated with normal saline. A gauze moistened with normal saline was applied to the burned area and secured with adhesive tape. The gauze was replaced the following day by cleaning the wound with normal saline and giving adhesive tape again. In the group treated with 5% astaxanthin gel, after the temperature of the burned skin decreased below 30°C, the burn was cleaned using normal saline. Then, 0.5 grams 5% astaxanthin gel was applied to the wound before it was covered with gauze (Figure 1). The gauze was replaced the next day by cleaning the wound with normal saline, applying 5% astaxanthin gel, and then securing it with adhesive tape.

## Histology study

Histologic examination was conducted in the Histology Department at Universitas Brawijaya, Malang using the excisional biopsy method on days 2, 5, and 7. The same animal group was examined histopathologically on days 2, 5, and 7. Tissues were fixed in 10% formalin solution, then processed and cut into 3-5 µm-thick paraffin sections. The slides were stained with haematoxylin & eosin (H&E).

The stained slides were then scanned using the viewer for histological examination (OlyVIA) program (Figure 2). The results of the tissue scan were quantified using the Image J software, version 1.52a. Angiogenesis was assessed by counting the number of blood vessels, characterized by lumen with endothelial cell layers and erythrocyte cells within the lumen. Granulation tissue was examined from the wound surface to the point in the dermis where fibroblast proliferation ceases.<sup>16</sup>

## Data analysis

The study data were processed using computerized analysis with statistical product and service solution (IBM-SPSS) software version 25 with a significance level of 0.05 ( $p$ -value < 0.05) and a confidence level of 95% ( $\alpha$  = 0.05). The normality of data was assessed with Shapiro-Wilk test. Homogeneity of variance was performed using Levene's test. An unpaired t-test was performed to compare the groups treated with normal saline and 5% astaxanthin gel across different treatment periods. Pearson's correlation analysis was performed to examine the relationship between angiogenesis and granulation tissue.

## Result

### Baseline data

After a 7-day acclimatization period, the experimental animals were divided into six groups. The total number of experimental animals was 30, with each group consisting of 5 animals. Both the control group (normal saline) and the treatment group (5% astaxanthin gel) were evaluated on days 2, 5, and 7 after scald burn induction. One of the 30 experimental animals died on day 5 (the day of tissue collection) in group 5. The initial body weight showed no significant difference between the groups, ranging from 221.80 to 233.00 grams ( $p$ -value > 0.05). There was also no significant difference between body weight on days 2, 5, 7 in normal saline and astaxanthin group [227.80(9.73) vs 214.60(13.24); 203.80(11.52) vs 221.80(19.25); 208.80(16.84) vs 215.00(17.07), respectively ( $p$ -value > 0.05)]. The results of the homogeneity using Levene's test for initial body weight, body weight in the end experiment, angiogenesis and granulation tissue showed homogeneity ( $p$ -value > 0.05).

### Angiogenesis

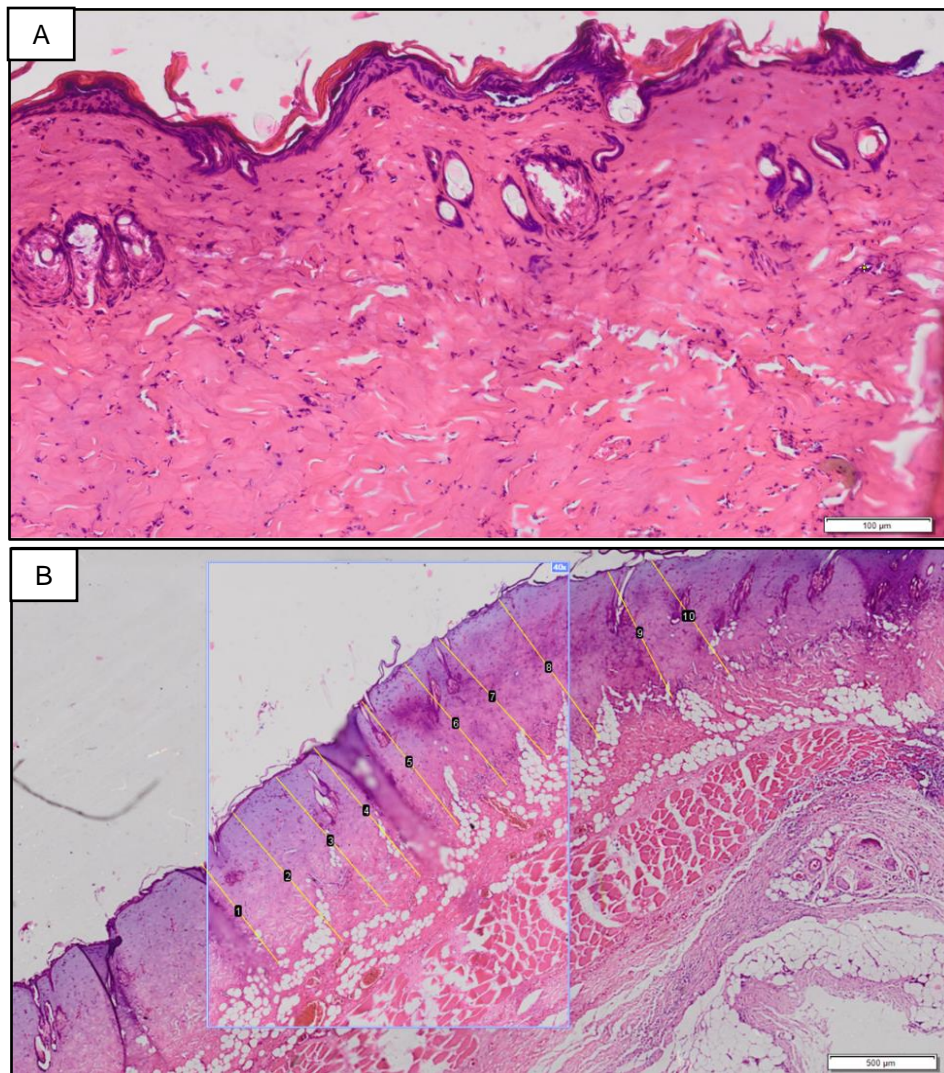
The level of angiogenesis was determined by the number of blood vessels formed in each group. The mean of angiogenesis level in normal saline group vs astaxanthin group on days 2, 5, and 7 showed no significant difference [4.87, 1.43, 3.17 vs 2.20, 4.53, 2.20, respectively]. However, the mean scores of angiogenesis scald burn in the groups treated with normal saline compared to those treated with 5% astaxanthin gel were significant on days 2 and 5 ( $p$ -value = 0.016 vs 0.025). During observation, the angiogenesis remained consistent with the initial data in the astaxanthin group (Table 1).

### Comparison of angiogenesis in groups treated with normal saline and 5% astaxanthin gel

The groups treated with normal saline showed the highest angiogenesis on day 2. By day 5, this had decreased to the lowest mean, with an increase noted on day 7. The group treated with 5% astaxanthin gel showed the same mean score of angiogenesis on day 2 and day 7 which was lower than on day 5 (Figure 3). This data showed that during the observation period, angiogenesis in the astaxanthin group remained consistent on day 2 and 7. In contrast, the normal saline group tended to show a decrease.



**Figure 1.** Scald Burn and Wound Care. A. Second-degree scald burns on the back of male Wistar rats. B. Treatment was administered to the groups with normal saline. C. Topical application in the group treated with 5% astaxanthin gel.



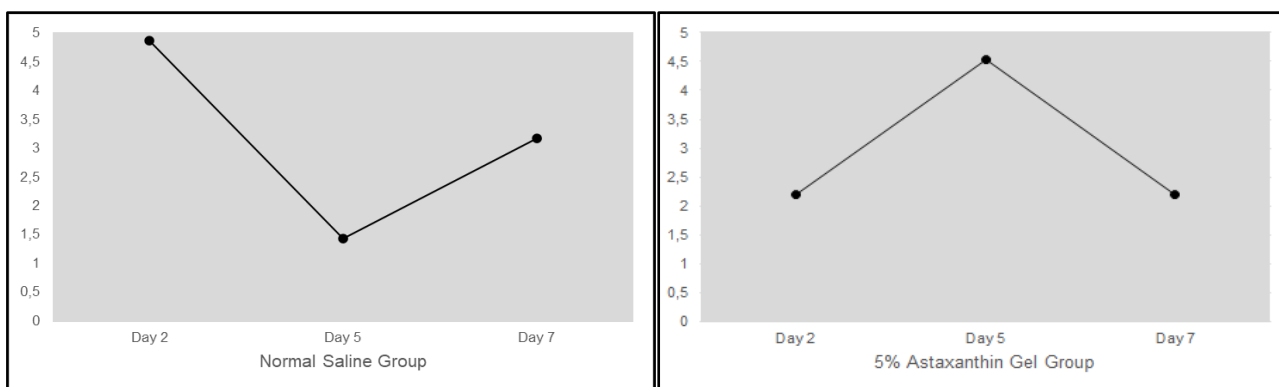
**Figure 2.** Histological Preparation.

A. Angiogenesis was measured from the number of blood vessels characterized by the presence of endothelial and erythrocyte cell layers in the lumen (x100,  $\rightarrow$ ). B. Granulation tissue was measured from the wound surface to the end of fibroblast proliferation (x100,  $\swarrow$  in  $\mu\text{m}$ )

**Table 1.** Comparison of Angiogenesis in Normal Saline and 5% Astaxanthin Gel Group

Group (n)	Mean (SD)	p-value
normal saline (n=5) vs 5% astaxanthin gel (n=5) day 2	4,87(1.45) vs 2,20(1.30)	0.016
normal saline (n=5) vs 5% astaxanthin gel (n=5) day 5	1,43(2.07) vs 4,53(1.43)	0.025
normal saline (n=5) vs 5% astaxanthin gel (n=5) day 7	3,17(2.46) vs 2.20(2.02)	0.517

Remarks: Unpaired t-test, if  $p < 0.05$  = significantly different. Data are presented in the form of: Mean (standard deviation). n=sample size



**Figure 3.** Comparison of Angiogenesis in Normal Saline vs 5% Astaxanthin Gel Group on Day 2, 5, 7

### Granulation tissue

There was no significant difference in the mean granulation tissue measurements between the normal saline group vs astaxanthin group on days 2, 5, and 7 [614.06  $\mu\text{m}$ , 406.26  $\mu\text{m}$ , 619.15  $\mu\text{m}$  vs 478.07  $\mu\text{m}$ , 510.30  $\mu\text{m}$ , 504.67  $\mu\text{m}$ , respectively]. Similarly, there was no significant difference between the mean score of granulation tissue 2 days after scald burn induction in the normal saline group compared to the 5% astaxanthin gel groups ( $p$ -value = 0.437). This trend continued to show on days 5 and 7 ( $p$ -value = 0.601 vs 0.339, respectively; Table 2).

The groups treated with normal saline showed greater granulation tissue than the astaxanthin group on day 2. However, on day 5, the astaxanthin group showed higher granulation tissue than the normal saline group. The granulation tissue of normal saline remained the same with initial data on day 2, unlike the astaxanthin group, which showed an increase on day 7 (Figure 4).

### Correlation of angiogenesis and tissue granulation

There was no significant relationship ( $p$ -value > 0.05) between angiogenesis and granulation tissue

in the two groups on day 2 ( $p$ -value = 0.415) and day 7 ( $p$ -value = 0.857). This showed that the level of angiogenesis did not determine the increase or decrease in granulation tissue of the normal saline and 5% astaxanthin gel groups on days 2 and 7 after the scald burn. Meanwhile, there was a significant relationship ( $p$ -value < 0.05) between angiogenesis and granulation tissue in the groups treated with normal saline and 5% astaxanthin gel on day 5 ( $r = 0.656$ ,  $p$ -value = 0.039). This suggests a strong positive correlation, implying that higher angiogenesis is associated with increased granulation tissue (Figure 5).

### Discussion

This study aimed to examine the effect of 5% astaxanthin gel on angiogenesis and granulation tissue using an animal model with second-degree scald burns. Two days after burn induction, angiogenesis in the groups treated with 5% astaxanthin gel was lower than those treated with normal saline ( $p$ -value < 0.05), but it was higher at day 5 ( $p$ -value < 0.05). Observations on day 2 indicated that 5% astaxanthin gel administration might not have a significant effect during the inflammatory phase. The reduced angiogenesis during the inflammatory phase is associated with the effect of astaxanthin in increasing the basic

fibroblast growth factor (bFGF), which is more prominent in the proliferative phase of burns. The bFGF factor has an important role in angiogenesis and granulation tissue formation.

At the initiation phase of angiogenesis, bFGF is stored in intact cells and extracellular matrix (ECM) is released from damaged tissue. The 5% astaxanthin gel effect tends to be more pronounced on day 5, coinciding with the vascular proliferative phase. Astaxanthin is assumed to induce vascular endothelial growth factor (VEGF), which regulates angiogenesis from proliferative to stabilization.<sup>6,17</sup> Seven days after burns, angiogenesis in the group treated with 5% astaxanthin gel began to decline. This indicated that the stabilization phase had been completed, and the suppression phase of angiogenesis had commenced. During this suppression phase, there is a decrease in hypoxia, a subsidence of inflammation, and a reduction in the level of growth factors.<sup>17,18</sup>

One of the indicators of successful wound healing is the formation of granulation tissue. A higher rate of granulation tissue formation is associated with a shorter wound healing process.<sup>6</sup> In this study, applying 5% astaxanthin gel had no significant

effect on second-degree scald burns compared to the group treated with normal saline ( $p$ -value > 0.05). However, granulation tissue in the group treated with 5% astaxanthin gel was higher on day 5 than the normal saline group.

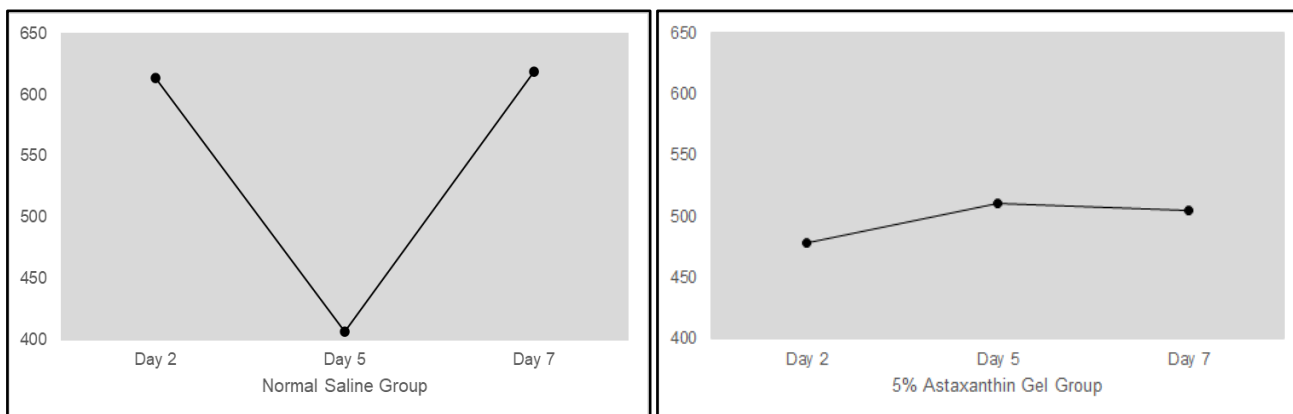
The observed increase in granulation tissue in the group treated with 5% astaxanthin gel is thought to be due to the upregulation of bFGF mRNA expression, contributing to the acceleration of wound closure.<sup>7,18</sup> As the angiogenesis process proceeds into the remodeling phase, granulation tissue decline indicates tissue recovery.<sup>6</sup> Subsequently, pericytes stabilize endothelial cells by releasing activated transforming growth factor-beta (TGF- $\beta$ ), which inhibits vascular proliferation.<sup>18,19</sup> This is in line with the data obtained from this study, the remodeling phase appeared to commence on day 7, as evidenced by a reduction in granulation tissue in the group treated with 5% astaxanthin gel.

A low ROS level is required to initiate angiogenesis, as ROS signaling regulates the formation of new blood vessels. Astaxanthin can suppress ROS and modulate its levels accordingly to activate physiological angiogenesis via the programmed Wnt/ $\beta$ -catenin signaling pathway.<sup>20,21</sup>

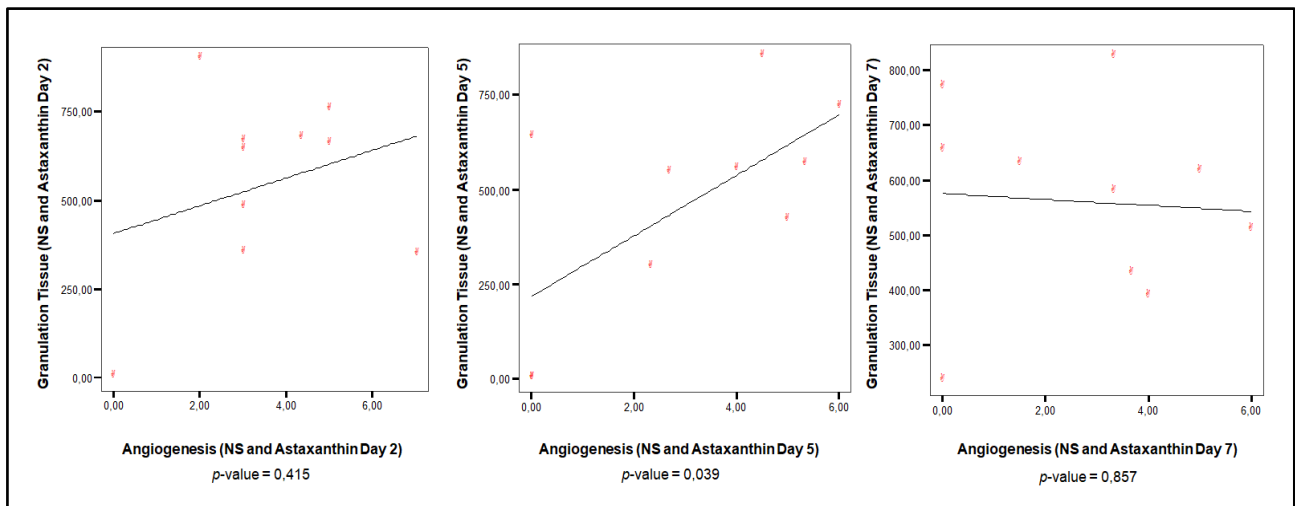
**Table 2.** Comparison of Granulation Tissue in Normal Saline and 5% Astaxanthin Gel Group

Group (n)	Mean (SD), $\mu$ m	$p$ -value
normal saline (n=5) vs 5% astaxanthin gel (n=5) day 2	614.06(156.46) vs 478.07(337.42)	0.437
normal saline (n=5) vs 5% astaxanthin gel (n=5) day 5	406.26(387.33) vs 510.30(159.78)	0.601
normal saline (n=5) vs 5% astaxanthin gel (n=5) day 7	619.15(95.09) vs 504.67(232.89)	0.339

Remarks: Unpaired t-test, if  $p < 0.05$  = significantly different. Data are presented in the form of: Mean (standard deviation). n=sample size.



**Figure 4.** Comparison of Granulation Tissue in Normal Saline and 5% Astaxanthin Gel Group on day 2, 5, 7



**Figure 5.** Graphic of Linearity between Angiogenesis and Granulation Tissue in Normal Saline and Astaxanthin Group  
NS: Normal Saline

In this study, the granulation tissue in the group treated with 5% astaxanthin gel was not significantly different from the group treated with normal saline. It is presumed that the ability of astaxanthin to regulate ROS, which maximizes the initiation of angiogenesis, was not optimal. This suboptimal initiation phase might be related to the inadequate dose or concentration of the astaxanthin formulation to fight free radicals effectively. In addition, it may be due to ROS levels exceeding the antioxidant capacity provided.

The correlation between angiogenesis and granulation tissue is concurrent and important in wound healing. Angiogenesis occurs first since it plays a role in initiating, maintaining, and supporting the newly formed granulation tissue with micronutrients. New granulation tissue was formed on the 3<sup>rd</sup> to 5<sup>th</sup>-day post-wound, where blood capillaries can be found on microscopic observation. Furthermore, vessel regression was observed at the end of the granulation phase at the 7-14<sup>th</sup> days.<sup>22,23</sup>

In this study, a positive correlation between angiogenesis and granulation tissue was observed on day 5 in both groups ( $p$ -value<0.05). Another finding from this study indicated an increase in angiogenesis and granulation tissue on day 7 in the group treated with normal saline. Ideally, during the final phase of wound healing (remodeling phase), angiogenesis and granulation tissue should be suppressed. The increased angiogenesis and granulation tissue at day 7 (remodeling phase) was probably associated with impaired suppression,

potentially caused by factors such as infection, hypoxic conditions, or inflammation.<sup>17,23</sup>

This study is the first to examine topical astaxanthin's application on burns.<sup>24</sup> In dermatology, topical treatment of astaxanthin extract from *Haematococcus pluvialis* has improved wound condition by providing protective effects through balanced oxidative actions. A balance between oxidative and antioxidative forces is necessary for optimal wound healing.<sup>25</sup> One study reported full-thickness dermal wound healing after receiving 78.9  $\mu$ M of topical astaxanthin for 15 days.<sup>18</sup>

Another study also reported that astaxanthin affects anti-aging, with various formulations examined for this purpose, including 5% astaxanthin-oleoresin, 0.8% astaxanthin algae extract, and a combination of 0.05% astaxanthin and 5% beta-glucan.<sup>23</sup> However, this study has several limitations. It did not evaluate astaxanthin at different percentages for effectively treating burns, nor did it examine systemic conditions that may affect wound healing.

In addition, wound care for the group treated with 5% astaxanthin gel was not conducted in a moist environment since the topical application was covered with dry gauze; thus, it was not as moist as the groups treated with normal saline. Moist environmental condition is beneficial for better wound healing.<sup>25</sup> Hence, a combination of saline compresses with the application of 5% astaxanthin gel could be considered for enhancing burn treatment.



## Conclusion

Based on the results obtained, we conclude that 5% astaxanthin gel has a more favourable effect on angiogenesis and granulation tissue formation than normal saline on day 5 following second-degree burn injuries. Therefore, the findings of this study indicate that topical application of 5% astaxanthin gel contributes positively during the proliferative phase of wound healing. Further research involving randomized controlled trials (RCT) is needed.

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## Author Contributions

All authors act as the guarantor of the manuscript. AR is the main investigator of this study. APY conceived of the study and participated in its design and coordination and helped to draft the manuscript. RHN participated in the conception, data acquisition, data interpretation, data analysis, statistical analysis of the study and writing of the study.

## Conflict of Interest

No conflict of interest.

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