ORIGINAL ARTICLE

The Effect of *Spirulina Platensis* Gel on Angiogenesis and Collagen Fiber Density in Gingival Wound Healing

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ABSTRACT

Introduction: Gingiva is a periodontal tissue that can be easily injured by several stimuli. The tissue responds to the injury through the wound healing process. The parameters of wound healing are angiogenesis and collagen fibers density. *Spirulina platensis* contains phycocyanin, saponin, and flavonoids which increase angiogenesis and collagen fibers density. This study aimed to determine the effect of 12% *Spirulina platensis* extract gel topical application on angiogenesis and collagen fibers density in the gingival wound healing process of Sprague Dawley rats. **Methods:** Thirty-six rats were given injury to the mandibular central incisor labial gingiva. These rats were divided into three groups: the treatment group (12% *Spirulina platensis* extract), the positive control group (Aloclair™), and the negative control group (CMC-Na). These rats were sacrificed to make histological preparations with Hematoxylin-Eosin and Trichrome Mallory. The blood vessel and collagen fibers density were observed using a light microscope at 400x and 13x40 magnification, respectively. Data were analyzed using the Two-Way ANOVA test and the Post-Hoc LSD test. **Results:** The results showed that the treatment group had the highest number of blood vessels and collagen fibers density on all observation days. There was a statistically significant difference in the number of blood vessels and collagen fibers density between the treatment and positive control group also between the treatment and negative control group. **Conclusion:** This study concluded that the 12% *Spirulina platensis* extract gel topical application could increase angiogenesis and collagen fibers density in the gingival wound healing process of Sprague Dawley rats.

Keywords: *Spirulina platensis* extract, Gingival wound healing, Angiogenesis, Collagen fibers density

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INTRODUCTION

Gingiva is a periodontal tissue that covered the alveolar process and surrounds the tooth cervix. The continuity of gingiva can be disrupted by physical, chemical, electrical, and thermal stimuli which causes injury to the tissue (1). The body will give a wound healing response to produce new tissues through four phases, such as hemostasis, inflammatory, proliferation, and remodeling phases (2). Gingival wound healing parameters can be seen from the formation of new blood vessels (angiogenesis) and collagen fibers density (3, 4).

The wound healing process can occur naturally or accelerated by the use of medicine. Accelerated wound healing can prevent the transition of an acute wound to a chronic wound, reduce morbidity, and minimize the formation of scar tissue (5, 6). The use of synthetic drugs has a risk of side effect; thus, people are starting to utilize natural ingredients as an alternative to medicine due to minimum side effect and maximum therapeutic effect to produce effective healing (7).

One of the natural ingredients used to accelerate wound healing is *Spirulina platensis*, i.e., spiral-shaped blue-green algae often found in tropical and subtropical climate regions with warm water (8). Indonesia is a tropical country with a warm climate that increases the risk of blooming algae phenomenon (9). Despite the impact of an imbalanced aquatic ecosystem, the abundant availability of *Spirulina platensis* has the potential to be utilized in dentistry as an alternative medicine to accelerate wound healing of the tissues in the oral cavity.

Spirulina platensis contains several active substances, which include heptadecane that acts as anti-bacterial,

phycocyanin as anti-oxidant and anti-inflammatory (10). *Spirulina platensis* contains phycocyanin which accounts for 4.5% of the total pigment (11). It can stimulate the expression of fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) which induced the proliferation and migration of endothelial cells, and increased hydroxyproline components in tissues, thus myofibroblasts will increase in the wound area and facilitate collagen deposition during the proliferation phase (12, 13). *Spirulina platensis* also contained flavonoids which are considered to stimulate the expression of VEGF and stimulate fibroblasts to synthesize collagen (14, 15). A previous study stated that 12% of *Spirulina platensis* extract gel can heal post-extraction wounds faster than 3% and 6% *Spirulina platensis* gel application (16).

A gingival wound is usually treated with Aloclair™ gel, which is a topical medicine contained Aloe vera extract, sodium hyaluronate, and polyvinylpyrrolidone in gel preparation widely used in dentistry to accelerate oral lesion and gingival wound healing (17). The gel is used as a medicine preparation due to ease of application, the formation of protective barrier, biodegradable and biocompatible properties, and had longer retention duration compared to other topical preparations (18).

An in vivo laboratory experiment of wound healing was conducted using animal models. Sprague Dawley rats were mostly used as animal models due to similar mucosal tissue structure with humans (19). This study was aimed to determine the effect of topical application of 12% *Spirulina platensis* extract gel on the angiogenesis and collagen fibers density in gingival wound healing of Sprague Dawley rats.

MATERIALS AND METHODS

Samples

This study was conducted using three ingredients, there are 12% *Spirulina platensis* extract gel, Aloclair™ gel, and 2% CMC-Na gel. The subjects were 36 male Sprague Dawley rats aged 2-3 months with 200-250 grams body weight. The rats were divided into three groups of application, each consisted of nine rats for treatment group (12% *Spirulina platensis* extract gel), positive control (Aloclair™ gel), and negative control group (2% CMC-Na gel). The ethical clearance from the Faculty of Dentistry Universitas Gadjah Mada was attained upon commencement of the study [Reference No: 00213/KKEP/FKG-UGM/EC/2019].

Treatment of Sprague Dawley *Rats*

All rats were anesthetized with an intramuscular injection of ketamine (50 mg/kg body weight) and xylazine (0.5-1.1 mg/kg body weight) on the upper left thigh muscle to provide a sedative effect. Afterward, a wound was made on the labial gingiva of the mandibular central incisor using a 3 mm punch biopsy. The wound was made exactly under the interdental of the mandibular central incisor. The treatment group was treated with 12% *Spirulina platensis* extract gel, positive control was treated with Aloclair™ gel, and negative control group was treated with 2% CMC-Na gel applied topically using a micro brush until covering all wound regions twice daily. The rats were sacrificed using ketamine overdose (three times the anaesthetic dose) on the 3rd, 5th, 7th, and 14th day to saw the angiogenesis process and the 3rd, 7th, and 14th day to saw the collagen fibers density. Histological preparation was taken from the wounded tissue and stained with Hematoxylin-eosin and Trichrome Mallory.

Microscopic observation and interpretation

The blood vessels were seen as a cavity filled with red-coloured erythrocytes and surrounded by endothelial cells in Hematoxylin-Eosin staining. The observation was performed using an Optilab Viewer microscope at 400x magnification in five fields of view. The mean number of blood vessels was obtained from three samples of each subject. Meanwhile, the collagen fibers were seen in blue in Trichrome Mallory staining. The observation of collagen fibers density was performed using 13x40 magnification in six fields of view. The observation results were captured and quantified using ImageJ software to obtain the data.

Statistical analysis

The data were tested using the Two-Way ANOVA test to determine the effect of materials and observation time on the number of blood vessels and collagen fibers density. The specific difference in the number of blood vessels and collagen fibers density between each group and observation time were identified with the Post-Hoc LSD test.

RESULTS

The mean number of blood vessels and the mean of collagen fibers density of each group is presented in Table I and II. Table I and Figure 1 showed that the blood vessels were starting to form on day 3 in each group. Afterwards, there was an increase in blood vessels on the 5th day and reached its peak on the 7th day. This increase was higher in the treatment group and positive control group. Each group showed a decrease in blood vessel number on the 14th day. The highest average blood vessel number was seen in the treatment group on all observation days. The positive control group had a lower number of blood vessels compared to the treatment group, but higher than the negative control group.

Table I : The mean and standard deviation of the number blood vessels in the treatment group, positive control group, and negative control group on the 3rd, 5th, 7th, and 14th days.

Group	n	Mean ± SD The Number of Blood Vessels			
		Day-3	Day-5	Day-7	Day-14
Treatment	12	3.93 ± 0.31	4.67 ± 0.31	6.27 ± 0.29	4.70 ± 0.35
Positive Control	12	2.83 ± 0.15	3.50 ± 0.26	5.00 ± 0.40	3.17 ± 0.61
Negative Control	12	2.20 ± 0.50	3.47 ± 0.40	3.60 ± 0.78	3.07 ± 0.06

Note :
n : number of sample
SD : standard deviation
Treatment : 12% *Spirulina platensis* extract gel
Positive Control : Aloclair™ gel
Negative Control : 2% CMC-Na gel

Table II : The mean and standard deviation of collagen fiber density in the treatment group, positive control group, and negative control group on the 3rd, 7th, and 14th days.

Groups	n	Mean ± SD Collagen Fiber Density (%)		
		Day-3	Day-7	Day-14
Treatment	9	40.17 ± 1.85	51.19 ± 0.81	58.25 ± 1.89
Positive Control	9	37.99 ± 1.86	45.45 ± 0.92	51.65 ± 0.82
Negative Control	9	29.47 ± 1.47	40.95 ± 1.37	48.06 ± 0.30

Note :
n : number of sample
SD : standard deviation
Treatment : 12% *Spirulina platensis* extract gel
Positive Control : Aloclair™ gel
Negative Control : 2% CMC-Na gel

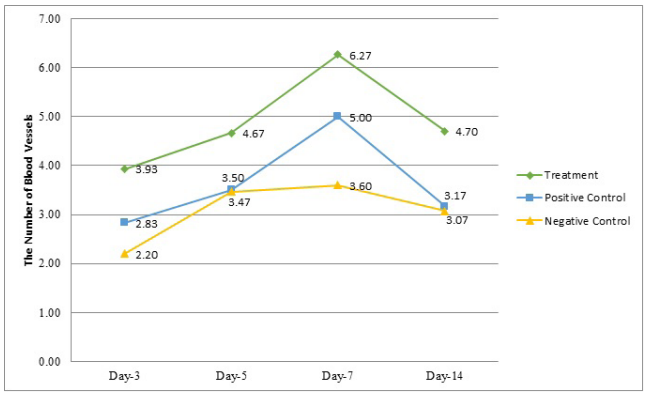

Figure 1 : Line chart of the mean number of blood vessels in the treatment group, positive control group, and negative control group on the 3rd, 5th, 7th, and 14th days. The observation of the number of blood vessels was performed using an Optilab Viewer microscope at 400x magnification in five fields of view. The number of blood vessels were counted using ImageRaster software. Data are given as the mean number of blood vessels.

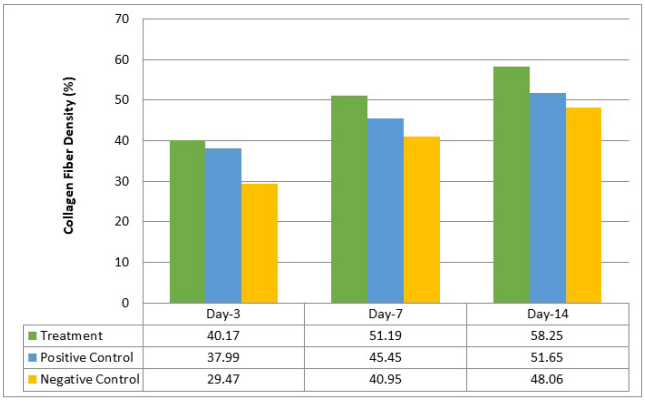

Figure 2 : Bar chart of the mean of collagen fibers density in the treatment group, positive control group, and negative control group on the 3rd, 7th, and 14th days. The observation of collagen fiber density was performed using 13x40 magnification in six fields of view. The results were captured, quantified, and analyzed using ImageJ software. Data are given as the mean of collagen fibers density (%).

Table II and Figure 2 showed that collagen fibers were formed on the 3rd day after application. The mean of collagen fibers density increased on the 7th and 14th days in the treatment group, positive control group, and negative control group. The highest collagen fibers density was seen on the 3rd, 7th, and 14th days in the treatment group. The 14th day was the day of the highest collagen fibers density in all three groups.

The results of the Shapiro-Wilk test showed p-values greater than 0.05, which indicated that the data were normally distributed. Levene's homogeneity test result showed significance value of 0.091 ($p>0.05$) for the number of blood vessel and 0.081 ($p>0.05$) for the collagen fibers density. These showed that the tested data had homogenous variance within each group.

After confirming normal distribution and homogenous data, the Two-Way ANOVA test was conducted. The number of blood vessels and collagen fibers density between the ingredients showed a statistically significant difference ($p < 0.05$). This means that the administration of ingredients significantly affects the number of blood vessels and collagen fibers density of the gingiva. The results of Two-Way ANOVA test also showed a statistically significant difference in the number of blood vessels and collagen fibers density between observation duration ($p<0.05$). This means that observation duration affected the number of blood vessels and collagen fibers density in the gingiva of Sprague Dawley rats. Interaction of the ingredients and observation duration showed a statistically significant difference ($p<0.05$) which means that interaction of the ingredients and observation duration significantly affects the number of blood vessels and collagen fibers density in the gingiva of Sprague Dawley rats.

Based on the results of the Post-Hoc LSD test (table III), there were statistically significant differences in the number of blood vessels ($p<0.05$) between the treatment group and positive control group, and between the treatment group and negative control group on all observation days. Meanwhile, there were several insignificant differences in the number of blood vessels between the positive control group and negative control group on the 3rd, 5th, and 14th days. There were several insignificant differences in the number of blood vessel ($p>0.05$) between the 3rd day treatment group and the 5th day positive control group, and between the 5th day treatment group and the 7th day positive control group.

Table III : Summary of Post-Hoc LSD test results of the number of blood vessels in the treatment group, positive control group, and negative control group on the 3rd, 5th, 7th, and 14th days.

T		Day-3			Day-5			Day-7			Day-14		
		PC	NC	T	PC	NC	T	PC	NC	T	PC	NC	T
Day-3	T		0.003*	0.000*	0.127	0.211	0.179	0.000*	0.004*	0.332	0.032*	0.032*	0.017*
	PC			0.072	0.000*	0.059	0.072	0.000*	0.000*	0.032*	0.000*	0.332	0.495
	NC				0.000*	0.001*	0.001*	0.000*	0.000*	0.000*	0.000*	0.008*	0.017*
Day-5	T					0.008*	0.007*	0.000*	0.127	0.017*	0.495	0.001*	0.000*
	PC						0.922	0.000*	0.000*	0.769	0.002*	0.332	0.211
	NC							0.000*	0.000*	0.696	0.001*	0.382	0.247
Day-7	T								0.001*	0.000*	0.000*	0.000*	0.000*
	PC									0.000*	0.382	0.000*	0.000*
	NC										0.003*	0.211	0.127
Day-14	T											0.000*	0.000*
	PC												0.769
	NC												

Note : (*) : Significant ($p<0.05$)
T : 12% *Spirulina platensis* extract gel group PC : Aloclair™ gel group
NC : 2% CMC-Na 2% group

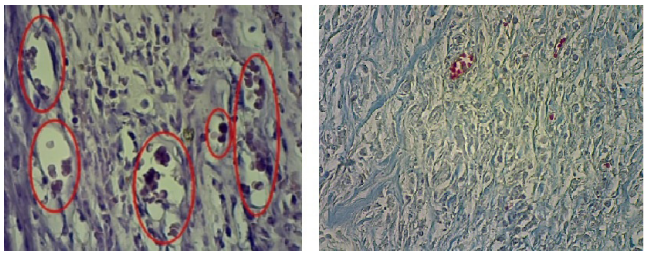

Figure 3 : Microscopic picture of blood vessels with Hematoxilin-Eosin staining at the time of observation on the 3rd day in the treatment group (left) and collagen fibers density with Trichrome Mallory staining at the time of observation on the 14th day in the treatment group (right).

Based on the results of the Post-Hoc LSD test (table IV), most of the data showed statistically significant differences ($p<0.05$). The results showed significant differences between observation duration of 3rd, 7th, and 14th days in each group. However, there were also several insignificant differences between the 3rd day positive control group and the 3rd day treatment group, the 7th day negative control group and the 3rd day treatment group, and the 7th day treatment group and the 14th day positive control group.

DISCUSSION

Indicators in gingival wound healing include an increase in the number of blood vessels and an increase in the density of collagen fibers. Based on the results of this study, the number of blood vessels started to form on the 3rd day and continue to increase until they reach a peak on the 7th day, but it decreased on the 14th day. Meanwhile, collagen fibers density was already formed on the 3rd day and increased on the 7th and 14th days in all groups. The results showed a statistically significant difference in the number of blood vessels and collagen fibers density between observation duration. This means that observation duration affected the number of blood vessels and collagen fibers density. This was following other research stated that the formation of blood vessels begun on the 3rd day and increased to reached its peak on the 7th day and decreased on the 14th day (20). Meanwhile, collagen fibers were already formed on the 3rd day after wound formation. During the proliferation phase, collagen fibers were synthesized more by fibroblasts and would be evident on the 7th day. By the 14th day, the collagen fibers would appear denser and thicker (21, 2).

The number of blood vessels and collagen fibers density between groups showed statistically significant differences. These mean that application ingredients affected the number of blood vessels and collagen fibers density. Each application ingredient had active substances that affect the number of blood vessels and collagen fibers density. CMC-Na gel is a gelling agent, which comprises physiological inert polymer chains and only act as a gelling agent in medicine, cosmetics, and food products (22). The inert property causes CMC-Na gel to not have a pharmacological effect on the number of blood vessels, compared to other ingredients. Aloclair™ gel contains sodium hyaluronate, and polyvinylpyrrolidone which can aid in the wound healing process (17). *Spirulina platensis* had phycocyanin, flavonoid, saponin, and triterpenoid which can accelerate wound healing (13, 15).

The results of the Post-Hoc LSD test on the number of blood vessels on the 3rd and 5th days showed statistically significant differences between the treatment group and the positive control group. The same can be seen between the treatment group and the negative control group. The results were due to the difference in the active substance contained in the ingredients. Phycocyanin contained in 12% *Spirulina platensis* extract gel supported the expression of bFGF as the primary proangiogenic factor since the first 3-5 days after wound formation. Phycocyanin stimulated the expression of bFGF through the uPA (urokinase-type plasminogen activator) pathway by degrading matrices. This showed that the application of 12% *Spirulina platensis* extract gel caused the release of proangiogenic factors, which lead to an angiogenic switch in the wound area (23). An angiogenic switch is caused by higher proangiogenic factor concentration compared to the antiangiogenic factor, thus stimulating the activation of endothelial cells and sprouting of old blood vessels (24).

The results of the Post-Hoc LSD test on the number of blood vessels on the 7th day showed statistically significant differences between the treatment group and positive control group, and between the treatment group and negative control group. This was due to phycocyanin content in 12% *Spirulina platensis* extract gel, which could reduce ROS (reactive oxygen species) level to create controlled oxidative stress, thus stimulating the formation of new blood vessels in the process of gingival wound healing. Phycocyanin had a therapeutic effect that reduce the concentration of reactive oxygen species (ROS) and nuclear factor-kappa B, thus increasing angiogenesis process and inhibiting the activation of caspase-3, which acts in the apoptosis of endothelial cells (25). The ability to create controlled oxidative stress is shown by the ability of phycocyanin in inhibiting the activity of NADPH oxidase, which is the main source of free radicals (26).

There was a significant difference between the treatment group and positive control group, and between the treatment group and the negative control

group in the number of blood vessels on the 14th day. This was due to the antioxidant effect from phycocyanin which stimulated the expression of VEGF in the angiogenesis process (27).

Insignificant differences of blood vessel number were found between the 3rd day treatment group and the 5th day positive control group, and between the 5th day treatment group and the 7th day positive control group. It can be explained by the phycocyanin content in 12% *Spirulina platensis* extract gel that acted in increasing the expression of bFGF (23), for the first three days to the fifth day after wound formation. The effect was optimized by saponin and flavonoid, which stimulated the expression of the proangiogenic factor. This caused the faster formation of blood vessels in the treatment group on the 3rd day and the 5th day, in which the positive control group only achieved on the 5th and 7th days.
Most groups and observation durations had significant differences, except several groups. Collagen fibers density on the 3rd day showed a statistically significant difference between the negative control group and the positive control group, and between negative control group and treatment group. However, the results were not significant between the positive control group and the treatment group. This may be due to the formation of new collagen fibers on the 3rd day. The active substances from Aloclair™ gel and 12% *Spirulina platensis* extract gel had started working effectively to synthesize collagen fibers, thus there was a insignificant difference on the 3rd day. On the 7th and 14th days, there were statistically significant differences between all groups, because the wound healing process was already effective physiologically.

The results also showed an insignificant difference between the 7th day negative control group and the 3rd day treatment group, and between the 7th day treatment group and the 14th day positive control group. This was due to the difference of active substances between the ingredients. The difference between the 7th day negative control group and the 3rd day treatment group was due to 2% CMC-Na gel contained in the negative control group had no therapeutic effect on the tissue. Meanwhile, *Spirulina platensis* extract gel had phycocyanin, flavonoid, and triterpenoid which can increase collagen fibers density in the wound healing process (28, 13, 15). Therefore, collagen fibers density that was seen on the 3rd day in the treatment group was achieved on the 7th day in the negative control group. This showed that the formation of collagen fibers was faster in the treatment group than in the negative control group. The 7th day treatment group and the 14th day positive control group also showed an insignificant difference. The difference of active substances became the causative factor. Thus, collagen fibers density that was seen on the 7th day in the treatment group, was seen on the 14th day in the positive control group. Aloclair™ gel contains Aloe vera that affect the growth factor of fibroblast and increase the synthesis of collagen fibers (29, 28). *Spirulina platensis* extract gel has phycocyanin, which can increase the synthesis and deposition of collagen fibers. It can increase the hydroxyproline ability in tissue, thus increasing myofibroblast and facilitate collagen deposition in the wound area (13). It can also increase the proliferation of fibroblasts through the cyclin-dependent kinase pathway (cdK1 and cdK2) and increase the migration of fibroblast through the uPA pathway (urokinase-type plasminogen activator) (28). The increase of fibroblasts leads to an increase in collagen fibers synthesis in the wound area (21). Flavonoid contained in *Spirulina platensis* can induce the production of transforming growth factor-β (TGF-β), which induces fibroblast to synthesize collagen fibers on the wound healing area (30). Triterpenoid can improve the proliferation of fibroblasts in the wound area and induce the synthesis of collagen fibers by fibroblasts through the activation of TGF-β (31). Phycocyanin and triterpenoid in *Spirulina platensis* that cannot be found in Aloclair™ gel causes the faster formation of collagen fibers.

Collagen fibers kept increasing each day, marked by denser collagen fibers compared to previous days. Wound healing entered the proliferation phase on the 3rd day, but did not rule out the possibility of an on-going inflammatory phase. There were only a few collagens on the 3rd day due to few numbers of fibroblasts. The proliferation phase kept increasing each day along with denser collagen fibers synthesized by fibroblasts induced by TGF-β of macrophages and fibroblasts. Collagen deposition in this phase was still randomly arranged. On day 14, the arrangement of collagen fibers appeared denser and more regular, due to reorganization of collagen fibers and collagen cross-linking in the remodeling phase which provided strength and density in tissues undergoing wound healing process (21, 2).

Based on the results of this study, 12% of *Spirulina platensis* had a higher effect on angiogenesis and collagen fibers density in the gingival wound healing process in Sprague Dawley rats compared to negative control and positive control groups. The formation of blood vessels was faster in the treatment group on the 3rd and 5th days, which was achieved by a positive control group on the 5th and 7th days. Meanwhile, collagen fibers density that was achieved on the 7th day in the treatment group, was only achieved on the 14th day in the positive control group. This showed that 12% of *Spirulina platensis* extract gel improved angiogenesis and collagen fibers density faster than the positive control group. This concludes that 12%

of *Spirulina platensis* extract gel can be used as an alternative medicine for gingival wound healing. In this study, there are several limitations, such as not able to determine the active compounds contained in the *Spirulina platensis* extract gel that affect angiogenesis and collagen fiber density in the gingival wound healing process. Therefore, it is necessary to conduct further tests on the compounds contained in the *Spirulina platensis* extract gel.

CONCLUSION

This study concludes that 12% *Spirulina platensis* extract gel topical application could increase angiogenesis and collagen fibers density in the gingival wound healing process of Sprague Dawley rats.

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