

## The Effect of a combination of 12% spirulina and 20% chitosan on macrophage, PMN, and lymphocyte cell expressions in post extraction wound

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### ABSTRACT

**Background:** Tooth extraction is the ultimate treatment option for defective teeth followed by the need for dentures. Inflammation is one phase of the healing process that should be minimized in order to preserve alveolar bone for denture support. Macrophage, PMN and lymphocyte cells are indicators of acute inflammation. Spirulina and chitosan are natural compounds with the potential to be anti-inflammatory agents. **Purpose:** This study aimed to determine macrophage, PMN and lymphocyte cells of animal models treated with a combination of 12% spirulina and 20% chitosan on the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> post-extraction day. **Methods:** Animal models were randomly divided into control (K) and treatment (P) groups. Each group was further divided into three subgroups (KI, KII, KIII and PI, PII, PIII). The post-extraction sockets of the control group animals were then filled with CMC Na 3%. Meanwhile, the post-extraction sockets of the treatment group members were filled with a combination of 12% spirulina and 20% chitosan. Subsequently, the number of PMN, macrophage and lymphocyte cells was analyzed by means of HE analysis on the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> days. Statistical analysis was then performed using a T-test. **Results:** There was a decrease in PMN cells and an increase in macrophage and lymphocyte cells on Days 1, 2 and 3. **Conclusion:** It can be concluded that a combination of 12% spirulina and 20% chitosan can not only decrease PMN cells, but can also increase macrophage and lymphocyte cells on day 1, 2 and 3 after tooth extraction.

**Keywords:** spirulina; chitosan; inflammation; PMN; macrophage; lymphocyte

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### INTRODUCTION

Tooth loss is one of dental health problem. Its prevalence in Indonesia, according to RISKESDAS data in 2013, was 14.51% within the 45-54 years age group, 25.02% in the 55-64 years age group, and 43.79% in the >65 years age group.<sup>1</sup> Tooth loss can occur due to extraction which can increase injury to dental tissues resulting in an acute inflammatory reaction as well as inflammatory cell infiltration, such as polymorphonuclear (PMN), macrophages, and lymphocytes.<sup>2</sup> Clinical inflammatory reaction is usually indicated by edema, redness, and pain.<sup>3</sup> The inflammatory phase commonly lasts from the point of injury to Day 6 after its occurrence.<sup>4</sup> Thus, in order to maintain the alveolar

bone while promoting prosthetic restoration, biomaterial is required to eliminate the inflammatory process and accelerate the post-extraction healing process.

Recently, the use of natural materials in accelerating the wound healing process has been widely studied since they are safer than synthetic materials. One of the natural ingredients that have many benefits for the wound healing process is spirulina - a greenish-green algae containing C-phycoyanin, B-carotenoids, vitamin E, zinc, and other components useful to the human body. C-phycoyanin is even considered to have an anti-inflammatory and antioxidant components.<sup>5,6</sup>

Another natural ingredient in the healing process that is commonly studied is chitosan, a polymer of deacetylated

chitin. Chitin is a copolymer of N-acetyl-d-glucosamine and D-glucosamine bound by  $\beta$ - (1-4) glycosidic bonds. Chitin and chitosan can both be found in aquatic and terrestrial organisms. Chitosan can currently be obtained via the food industry through the processing of waste derived from shrimps, lobsters, crabs, and squids.<sup>7</sup> Chitosan is widely used as one of ingredients for drug delivery systems, wound healing processes, and orthopedic implants, while also being known to increase the activities of immune cells, inflammatory cells and angioendothelial cells. Chitosan oligosaccharides have anti-inflammatory properties since chitosan can inhibit the production of tumor necrotizing factor- $\alpha$  (TNF- $\alpha$ ) in inflammation stimulated by lipopolysaccharide (LPS).<sup>6</sup> Other studies have also shown that chitosan can reduce the inflammation associated with allergic responses by inhibiting the secretion of interleukin-8 (IL-8) and TNF- $\alpha$ .<sup>7</sup>

Some cases in the field of prosthodontics, such as immediate denture and ovate pontik installation, also require a more rapid wound healing process. Consequently, a shorter treatment that can lead to optimal results for patient comfort is necessary. Considerable previous research into the effects of spirulina and chitosan induction on collagen, osteoblast, and osteoclast cells in animal models has been conducted. The results of past investigations have shown that a combination of 12% spirulina and 20% chitosan can not only increase collagen and osteoblast expressions, but can also decrease osteoclast expression. As a result, it can be said that spirulina and chitosan has the potential to promote the bone healing process.<sup>8</sup> The effects of spirulina and chitosan in this regard have actually already been studied, unlike the effects of a combination of spirulina and chitosan on inflammatory cells. Therefore, this research aimed to reveal the effects of a combination of 12% spirulina and 20% chitosan on PMN, macrophage and lymphocyte cells in animal models on day 1, 2 and 3 after extraction.

## MATERIALS AND METHODS

This investigation reported here constituted laboratory-based experimental research incorporating a post test-only control group design and using male *Cavia cobaya* specimens (n = 42) weighing 300-350 grams and aged 3-3.5 months. The research passed an ethical test performed by the Faculty of Dental Medicine, Universitas Airlangga (no. 110/KKEPK.FKG/VII/2016).

The animal specimens were subsequently divided into two groups, namely; control and treatment. The control (K) and treatment (P) groups were each sub-divided into three sub-groups referred to as KI, KII, KIII, PI, PII, and PIII. The roman numerals I, II and III represent day 1, 2, and 3 after the specimens had been terminated.

Thereafter, the mandibular incisors of the members of all groups were extracted under ketamine anastesi (Ketalar, PT Pfizer, Jakarta, Indonesia) at a dose of 40 mg/kgBW. After the extraction, the sockets of the KI, KII, and KIII control groups were filled with 3% sodium-carboxymethyl cellulose natrium (CMC Na). Meanwhile, the sockets of the treatment groups, PI, PII, and PIII, were filled with a combination of 3% CMC Na, 12% spirulina and 20% chitosan using a 0.1 cc syringe. The sockets were stitched with 3/0-size silk thread. After the treatment, these animals were returned to their cages. On the first day, members of the KI and PI groups were decapitated, a process repeated for the KII and PII groups on day 2, as well as the KIII and PIII groups on day 3. Mandible samples were then taken and fixed.

At that point, the mandibles were with 2.5% nitric acid for 2 days. Thereafter, the sagittal incisive socket area of the specimens was cut and soaked in a 10% buffered formalin for 24 hours. Mixed preparations were then performed by using eosin haematoxylin (HE) before PMN, macrophages, and lymphocytes on one-third of the sockets' area were observed using a light microscope (Nikon H600L®, Tokyo, Japan) at a magnification of 400x. The data obtained was then analyzed by means of a Saphiro Wilk test to analyze the data distribution, followed by an Independent t-test to identify the differences between the groups.

## RESULTS

The results of the observation of PMN, macrophage, and lymphocyte cells in the control and treatment groups can be seen in Table 1. Moreover, the results of HE staining in both groups are contained in Figure 1. The results of HE staining illustrate that PMN possessed a large cell picture, a nucleus featuring lobes (2-5 lobes), chromatin in a condensed nucleus and visible organelle in cytoplasm. They also depicted macrophages as having a large cell image and generally kidney-shaped, erratically located cell nuclei. Furthermore, the cytoplasm cells were solid and appeared to be composed of a pink and purple granule,

**Table 1.** Mean and standard deviation of PMN, macrophage, and lymphocyte expressions

| No | Groups | n | PMN        | Macrophages | Lymphocytes |
|----|--------|---|------------|-------------|-------------|
| 1  | KI     | 7 | 41.85±5.87 | 1.43±0.53   | 3.00±0.82   |
| 2  | PI     | 7 | 32.00±3.26 | 5.57±1.51   | 12.00±2.00  |
| 3  | KII    | 7 | 35.29±2.81 | 2.00±1.15   | 8.28±2.28   |
| 4  | PII    | 7 | 11.71±1.60 | 19.71±1.98  | 21.43±2.37  |
| 5  | KIII   | 7 | 20.57±3.69 | 15.57±1.72  | 19.14±2.41  |
| 6  | PIII   | 7 | 15.14±2.41 | 20.57±2.70  | 22.43±3.31  |

whereas the lymphocytes had a circular or spherical nucleus cell with dark blue chromatin and a surrounding thin, light blue cytoplasm.

Subsequently, before a t-test analysis was performed, normality test, Saphiro Wilk test was conducted. Results of the Saphiro Wilk test showed that all data were normally distributed because p value was more than 0.05. Independent t-test then was carried out. Results of the independent t-test indicated that the p values of PMN cells between KI and PI groups, between KII and PIII groups, and between KIII and PIII groups were 0.020, 0.000, and 0.007, respectively. The results of the independent t-test also showed that the p values of macrophages between KI and PI groups, between KII and PII groups, and between KIII and PIII groups were 0.000, 0.000, and 0.001, sequentially. Meanwhile, the p values of lymphocytes between KI and PI groups, between KII and PII groups, and between KIII and PIII groups were 0.000, 0.000, and 0.055, respectively. Almost all of the independent t-test results in the control groups compared with the treatment groups indicated significant increase macrophages and lymphocytes differences ( $p < 0.05$ ), except between group PMN significant decrease ( $p > 0.05$ ). Thus, it can be said that no significant difference existed between the two groups.

## DISCUSSION

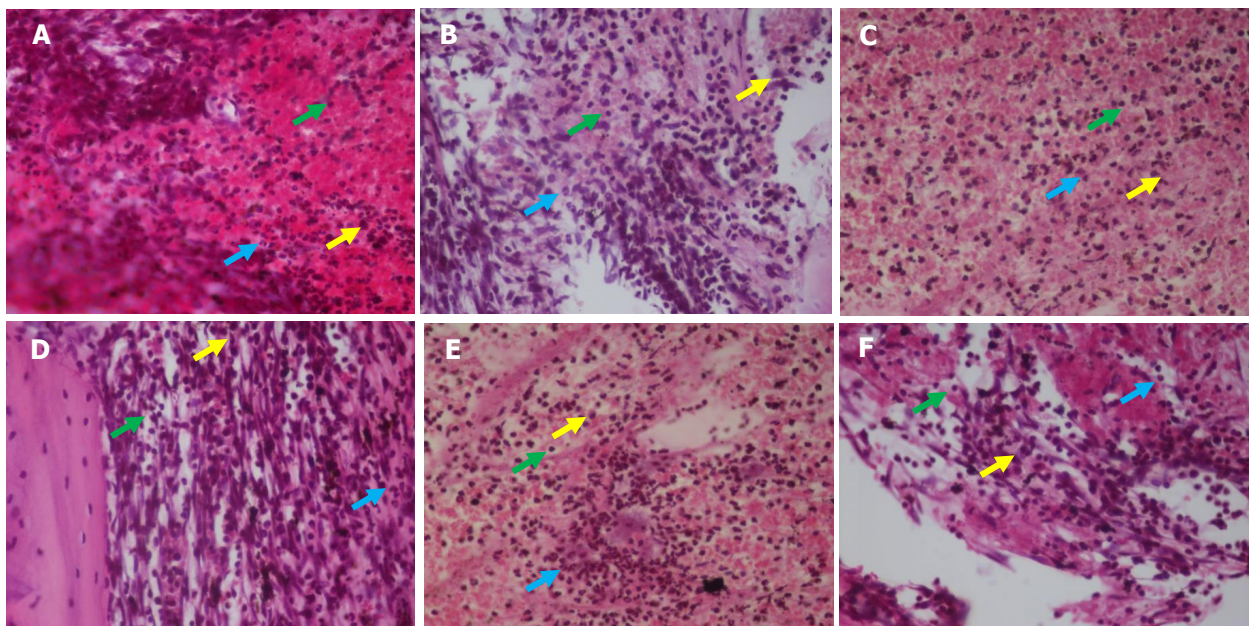
The presence of PMN cells is very important as an indicator of the wound healing process since PMN cells are the first to appear in the acute inflammatory phase.<sup>9</sup> PMN cells are cellular defenses that play an active role in the process of bacterial destruction through endothelial

adherens, chemotaxis and phagocytosis. Furthermore, PMN cells are able to move actively, and in a short period of time can collect in large numbers in the wound area. PMN cells have a lifespan of 1-3 days in connective tissue.<sup>10</sup>

Another cell that plays a role in the inflammatory process is the macrophage cell which carries out several functions in the wound healing process, such as producing collagenase and elastase enzymes, generating cytokines, facilitating phagocytosis and angiogenesis processes, as well as stimulating granulation tissue formation in the proliferative phase.<sup>11</sup> Macrophage cells in the inflammatory process can be distinguished by the origin of the tissue macrophage resident and monocytes undergoing differentiation.<sup>12</sup> Monocytes in the blood vessels will be transported toward inflammatory tissues due to chemotaxis resulting from the response to chemoattractant. Chemoattractant consists partly of kemokin, a protein (8-14 kDa) that regulates cell travel through interaction with a 7-transmembrane subset G-protein pair receptor.<sup>13</sup>

Similarly, lymphocyte cells play a role in the inflammatory process. Lymphocytes result in both immune-mediated inflammation caused by infectious agents and non-immune inflammation. T and B lymphocytes migrate to the inflammatory area and direct neutrophils and other leukocytes.<sup>14</sup> In the remodeling process, when the wound has been closed and the local infection has subsided, the leukocyte substance most often found in the injured tissue is that of T cell which acts as an adaptive immune response cell.<sup>6</sup>

Results of the research on day 1 revealed that the presence of PMN cells in the control group was higher than in the treatment group. This occurred because phycocyanin and  $\beta$ -carotene contained in spirulina can decrease the



**Figure 1.** Inflammatory cells in the tooth extraction sockets of *Cavia cobaya* animals in Group KI (A), Group PI (B), Group KII (C), Group PII (D), Group KIII (E), and Group PIII (F). (blue arrows: PMN, yellow arrows: macrophage cells, green arrows: lymphocytes).

production of proinflammatory cytokines, TNF- $\alpha$  and IL-1 $\beta$ .  $\beta$ -carotene in spirulina also has an anti-inflammatory effect through resistance to the production of nitric oxide and prostaglandin E2. Furthermore,  $\beta$ -carotene also inhibits the expression of INOS, COX2, TNF $\alpha$  and IL1 $\beta$ . Suppression of inflammatory mediators is due to NF- $\kappa$ B inhibition that restricts the nuclear translocation of subunits NF- $\kappa$ B p65.<sup>15</sup>

As a result, anti-inflammatory activity of spirulina can decrease the number of PMNs in the lesion site. TNF- $\alpha$  and IL-1 $\beta$  can also facilitate the movement of PMN cells to the site of the lesion and spur the production of endothelial adherens.<sup>16</sup> It is intended that PMN cells easily pass through the gap between endothelial cells in capillary blood vessels to eliminate bacteria, so the reduction of proinflammatory cytokines can, in turn, lead to a decrease in the production of PMN cells.<sup>16</sup>

In addition, on the first day after dental extraction, the average number of PMN cells in Group KI was the highest compared with the other control groups (K II and K III) and the treatment groups on the other days (P II and P III) because PMN cells had already been active and assembled at a large number of lesion sites very rapidly, i.e. within hours.<sup>17</sup> PMN cells are highly reactive to chemotactic products in the form of proteins produced by bacteria.<sup>16</sup>

Moreover, the results of calculating macrophage cells on day 1 indicated that the treatment group (P I) had a higher number of such cells than the control group (K I). This may occur because the inflammatory cells that appear immediately post-incision are not only neutrophils, but also monocytes moving into the inflammatory area. However, the number of monocytes migrating to the wound area is not as high as that of neutrophils.<sup>18</sup> The combination of 12% spirulina and 20% chitosan also contains more synthetic C-phycocyanin components which execute a greater immunomodulatory function than when applied alone.<sup>19</sup>

Furthermore, the results of the independent t-test showed there to be a significant difference in the presence of lymphocytes between the K1 and P1 groups on the first post-extraction day. This may occur because phycocyanin pigments contained in spirulina may act as an anti-inflammatory by inhibiting proinflammatory cytokines, namely; TNF- $\alpha$  and IL-1 $\beta$ .<sup>15</sup> Chitosan also plays a role in increasing lymphocyte cells. A previous piece of research using mice orally induced with chitosan finds that the latter can stimulate the release of IL-10, IL-4, and TGF- $\beta$  mRNA expressions in gastric mucosa, CD3 + T lymphocytes in the spleen, as well as natural killer cells (NK) in intestinal intraepithelial lymphocytes.<sup>20</sup>

In addition, the number of PMN cells in Group PII, based on the results of the second day's observation, was lower than that in Group KII. The decrease in PMN cells on the second day was higher than on the first day due to the homeostasis process where the number of PMN cells produced in the bone marrow must be balanced with that having clearance which had already worked on the system within the body. The number of PMN cells produced

in the bone marrow can reach a post-injury maximum within 24-48 hours. On the other hand, the process of PMN cell clearance can reach its peak 48 hours after the occurrence of the lesion. The number of PMN cells will then decrease as the chronic inflammatory phase is entered. Clearance can also occur when the PMN cells extravasate into the peripheral tissues. A previous investigation into mice revealed that PMN cells can migrate back from peripheral tissue into the bloodstream through a process known as reverse transmigration.<sup>16</sup> This suggests that the extravasation of PMN cells does not necessarily lead to the clearance of tissue. The clearance process reaches its peak on the second day. Consequently, the anti-inflammatory effect of the combination of 12% spirulina and 20% chitosan will reduce the number of PMN cells on the second day to a greater extent than on the first day.<sup>16</sup>

The number of lymphocytes in Group PII, based on the results of the second day of observation, was higher than that in Group KII. This difference is due to spirulina being able to increase the number of lymphocytes. Previous research has even shown spirulina to have an immune modulatory effect on lymphocytes by significantly increasing IFN- $\gamma$  production.<sup>21</sup> The increased number of lymphocyte cells in the treatment group is also due to chitosan that exhibits biocompatible and biodegradable properties. The latter allow chitosan to be broken down into micromolecules so that they are easily absorbed by the body without causing toxicity, thereby enabling it to be used as an analgesic, anti-tumor, anti-microbial, anti-oxidant, and wound healing agent.<sup>22</sup> Chitosan also contains a N-acetyl-D-glucosamine unit, polysaccharide similar to glucan, which accelerates cytokine production in order to stimulate repair of affected tissue.<sup>23</sup>

The results of the third day's observation found that the number of PMN cells in Group PIII was lower than that in Group KIII. The number of PMN cells on day 3 was lower than that on Day 2. This happened because the number of PMN cells will usually decrease between day 3 and 7.<sup>18,24</sup> This reduction is essential to preventing further damage to healthy body tissue. The body responds to a reduction in the production of PMN cells in order not to damage other tissues because PMN cells issue anti-microbial products that can damage healthy tissue of the body.

The number of macrophages in Group PIII, based on the results of the third day's observation, was still higher than that in Group KIII. Nevertheless, there was no significant difference in the post-extraction number of macrophages between the treatment groups on day 2 and day 3. This may be due to the inflammatory process beginning to enter the resolution phase during which a reduction of chemokine by the mechanism of proteolysis and chemokine sequestration occurs.<sup>25</sup> Macrophages, as a result, begin to dominate the wound area from day 3 to day 7 after extraction.

The number of lymphocyte cells in the treatment group, based on the results of the third day's observation, appeared to increase compared with that of the control group. However, based on the results of the independent t-test, there was no significant difference in the number

of lymphocyte cells between the control group and the treatment group on day 3. During the chronic inflammatory process, the presence of lymphocyte can usually reach a peak between day 5 and day 10.<sup>15</sup> Thus, 12% spirulina and 20% chitosan are expected to act as anti-inflammatories. This insignificant difference between the control group and the treatment group is due to the chronic inflammatory process having begun to subside and entering the maturation process which leads to regeneration.

The phycocyanin and  $\beta$ -carotene substances contained in spirulina can be considered to be anti-inflammatory antioxidants that can accelerate the wound healing process. Phycocyanin, according to previous *in vitro* and *in vivo* research using rat-fed animals, can inhibit TNF- $\alpha$  inflammatory cytokine secretion and act as an antioxidant.<sup>26</sup> This strongly suggests that the anti-inflammatory activity of spirulina may cause TNF- $\alpha$  and IL-1 $\beta$  secretions to decrease.<sup>27</sup> Similarly, the antioxidants in  $\beta$ -carotene contained in spirulina can improve the wound healing process.<sup>6</sup> Chitin and chitosan are biopolymers that offer many benefits, including; a high level of biocompatibility, low toxicity, increased antibacterial activity and accelerated wound healing.<sup>28</sup> Chitosan can also stimulate PMN cells to chemotaxize the wound area due to the presence of IL-1, TNF- $\alpha$ , and bacterial products.<sup>29</sup>

In other words, spirulina and chitosan exert a synergistic effect when combined since chitosan plays a role in drug delivery, while spirulina has a therapeutic effect. Spirulina and chitosan can also interact intermolecularly to increase mechanical resistance. Both of these materials can even work together to provide more effective benefits than if applied alone.

In addition, a high degree of chitosan acetylation will improve the hydrophobic properties of chitosan. The hydrophilic component is capable of diffusing through the chitosan polymer into the outer medium to be absorbed by the body.<sup>22</sup> Finally, based on the above discussion, it can be concluded that 12% spirulina and 20% chitosan combination can not only decrease PMN expression, but also increase macrophages and lymphocytes in *cavia cobaya* animals on the first, second and third days after extraction.

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