The expression of TNF-α, IL-1β, and IL-10 in the diabetes mellitus condition induced by the combination of spirulina and chitosan

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The expression of TNF-α, IL-1β, and IL-10 in the diabetes mellitus condition induced by the combination of spirulina and chitosan

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ABSTRACT

Background: Rapid wound healing is needed after tooth extraction treatment so that prosthodontics treatment can be done immediately. However, systemic diseases, such as diabetes mellitus, can inhibit the wound healing process. The state of hyperglycemia in people with diabetes mellitus can interfere with the function of macrophages and neutrophils. This condition can cause an imbalance in the cytokines IL-1β, TNF-α and IL-10 that are produced by macrophages and thus will affect the wound healing process. Spirulina and chitosan are two natural substances that have been proven to have anti-inflammatory properties. This study was aimed to determine whether the combination of 12% spirulina and 20% chitosan can accelerate wound healing through its intervention in cytokines IL-1β, TNF-α and IL-10.

Method: The samples used were 36 Wistar rats suffering from diabetes mellitus and divided into control groups induced by CMC Na base gel and treatment groups induced by the combination of 12% spirulina and 20% chitosan in the socket. These 2 groups were further divided into groups terminated on one day, three days and seven days post-extraction.

Results: The amount of IL-1β and TNF-α in the treatment groups is lower than that of the control group. Meanwhile, the treatment group of IL-10 showed an increase in IL-10 levels.

Conclusion: The combination of 12% spirulina and 20% chitosan can accelerate wound healing. They are decreasing the amount of pro-inflammatory cytokines IL-1β and TNF-α as well as increasing the number of anti-inflammatory cytokines IL-10.

Keywords: spirulina, chitosan, TNF-α, IL-1β, IL-10
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INTRODUCTION

The International Diabetes Federation (IDF) states that as many as 8.8% of the world's population of around 425 million people suffer from Diabetes Mellitus (DM) in 2017. Indonesia is a country with the seventh prevalence of diabetes in the world with 10.3 million sufferers and 2045; it is estimated that DM sufferers in Indonesia will be as many as 16.7 million people.[1] Tooth extraction can be done on a diabetic patient if the blood glucose is controlled. However, in the case of uncontrolled diabetes, extraction can cause microvascular complications.[2] After the tooth extraction, prosthodontics treatment is needed to restore the oral function of the missing teeth. This treatment requires healthy and intact bone for the retention of dentures. However, a systemic abnormality in diabetes mellitus can inhibit the wound healing process after extraction. As a result, prosthodontics treatment will be hampered.[3] Wound healing disorders in people with diabetes are caused by impaired function of macrophage and neutrophil. An imbalance between M1 (classically activated macrophages) and M2 (alternatively activated macrophage) polarization in diabetics causes an increase in expression of pro-inflammatory cytokines such as IL-1β & TNF-α by M1. In addition, it can also decrease the anti-inflammatory cytokines such as IL-10 by M2.[4] Excessive production of pro-inflammatory cytokines and reduced the production of anti-inflammatory cytokines will result in a longer-lasting inflammatory process that can lead to uncontrolled tissue damage.[5] In this era, a bone graft is an excellent material to accelerate bone regeneration. A good bone graft material must be able to repair the damaged body tissues rapidly. It works through the process of osseointegration, osteogenesis, osteoinduction, and osteoconduction.[6] The type of bone graft commonly used, namely allograft and autograft, has several disadvantages. They are susceptible to infection, causing pain, being expensive and having low osseointegration ability so that bone graft material cannot integrate well with host body tissues.[7]

Lately, a lot of research has been done on the effects of materials derived from nature, such as spirulina and chitosan. Spirulina is a greenish-blue group of algae-derived from the Oscilatoriaceae family.[8] Spirulina is often used in the health and food industry

because of its amino acid, mineral, essential fatty acids, and antioxidants content. In addition, this species has also been tested to have no toxic effects on human organs. Some of the content of spirulina namely C-phycocyanin and β-carotene has been proved to have several pharmacological advantages such as anti-inflammatory, immunomodulatory, anti-oxidant and anti-cancer activity.[8] Chitosan is a derivative of chitin obtained from structural components in the crab shell, shrimp and fungi cell wall. Hydrolyzed form of chitosan namely N-acetyl-Dglucosamine oligomers (NACOS) and D-glucosamine oligomers (COS) have been shown to have biological effects such as anti-cancer and anti-inflammatory.[9] Based on previous research, COS can suppress lipopolysaccharide (LPS) through a mechanism involving NF-κB blockade so that the production of pro-inflammatory cytokines is inhibited.[10]

A study conducted by Salim. et al. (2015) has proven that the combination of 12% spirulina and 20% chitosan affect the wound healing process by increasing the number of osteoblasts and reducing the number of osteoclasts significantly. Besides, the synergistic combination between these 2 materials occurs because spirulina is able to act as a cross-linking agent, thereby increasing chitosan resistance and elasticity.[11] Based on the mentioned above, this study aims to determine the effect of the combination of 12% spirulina and 20% chitosan on cytokines IL-1β, TNF-α, and IL-10 in the faster-wound healing process

METHOD

This study was an experimental laboratory study using a post-test only control group design. Thirty-six male, 3 months old with a weight of ± 150 grams *Rattus norvegicus* were used as the experimental subjects. These experimental subjects were divided into 6 groups, K1 (control group euthanized 1 day after tooth extraction), K2 (control group euthanized 3 days after tooth extraction), K3 (control group euthanized 7 days after tooth extraction), P1 (treatment group euthanized 1 day after tooth extraction), P2 (treatment group euthanized 3 days after tooth extraction), and P3 (treatment group euthanized 7 days after tooth extraction).

Experimental animals were adapted for 5 days before the study started. The subjects were given standard food and drink *ad libitum*. Streptozotocin (STZ) was given to the subjects 20 mg/kg of weight intraperitoneally.[12] After STZ induction, a 10% sucrose solution or 10% dextrose solution was given to subjects to prevent hypoglycemic shock. STZ was given daily for 5 days.[13] Five days after the last day of STZ induction, subjects were tested for fasting blood glucose levels. If the fasting blood glucose level of the subject is above 150 mg/dl, the subject was considered as diabetes mellitus positive.[13] These subjects were then divided into 6 groups as mentioned. The left mandible incisive of each subject were extracted under 0,1 ml/kg of weight of ketamine for anaesthesia. 0,1 cc of 3% CMCNa was given to the post-extraction socket of every subject within the control groups using 1 ml syringe; then the sockets were sutured. 0,1 cc of combination gel of 12% spirulina and 20% chitosan in 3% CMCNa was given to the post-extraction socket of every subject within the treatment groups using 1 ml syringe, and then the sockets were sutured.[14] On the first, third, and seventh-day post tooth extraction, the subjects were euthanized. The mandible bone of each subject was taken and immersed in a buffer solution. The mandible was then made into histopathology slides. The slides were stained using ScyTek Laboratories immunohistochemistry staining with Santa Cruz Biotechnology monoclonal antibody. TNF-α, IL-10, and IL-1b on the 1/3 apex of the socket were counted using a light microscope with 1000x magnification using a counting technique with a graticule on 20 fields of view.[11]

The acquired data were tested using IBM SPSS Statistics Software. Shapiro –Wilk test was used to test the normality of the data and Levene's Statistic Test was used to test the homogeneity of the data. Homogeneous data was then analyzed using the Post-HocTukey HSD Test on K1 and P1, K2 and P2, and K3 and P3 groups to know the significant difference between each group while data that was not normally distributed was tested using Mann-Whitney Test.[11] Significance was set at $p<0.05$.

RESULTS

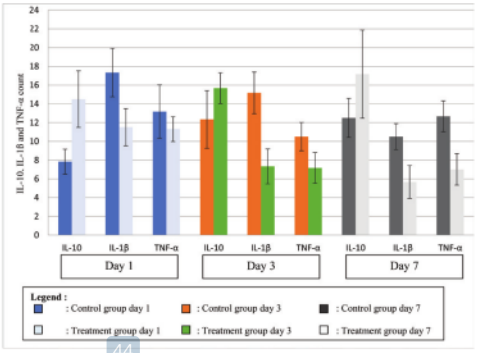

Figure 1 IL-10, IL-1β, and TNF-α count mean

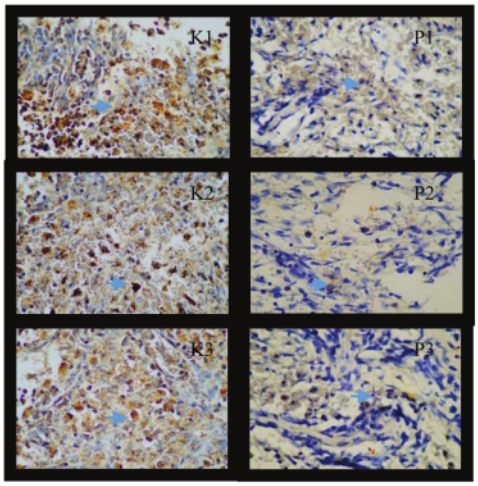

Figure 2 TNF-α histopathology expression on the macrophages of the post-extraction socket (1000x magnification).

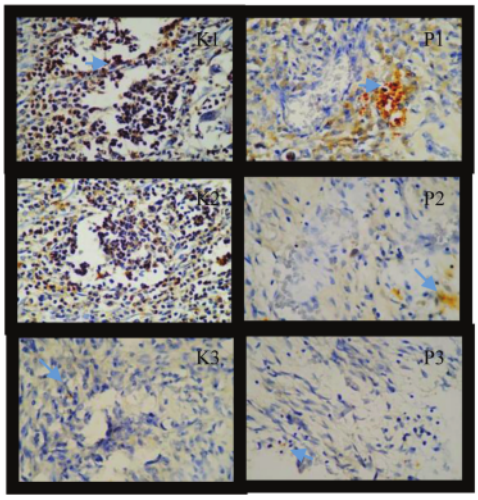

Figure 3 IL-1β histopathology expression on the macrophages of the post-extraction socket (1000x magnification).

DISCUSSION

Wound healing in patients with DM is slower than in non-diabetic patients. The previous study by Wu et al. describes that under DM conditions, there is an accumulation of advanced glycation end products (AGEs) and the amount of receptor for AGEs (RAGEs). Excessive AGE-RAGE interactions will increase transcription factor NF-κB activity.[15] As a critical transcription factor related to macrophages M1 activation, NF-κB regulates many pro-inflammatory genes expression including TNF-α and IL-1β.[16] High levels of pro-inflammatory cytokine also require a balanced mechanism. Therefore, the

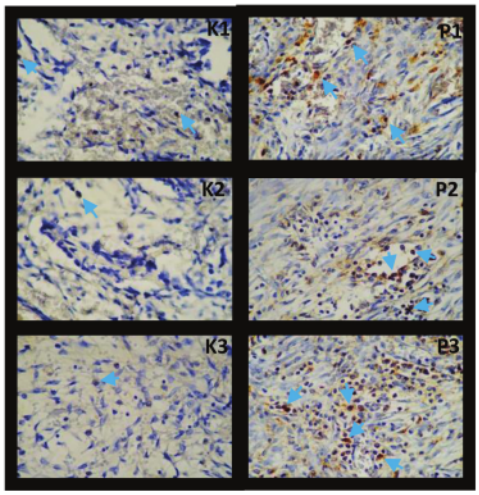

Figure 4 IL-10 histopathology expression on the macrophages of the post-extraction socket (1000x magnification).

body responds to balance the reaction of pro-inflammatory cytokines by releasing some anti-inflammatory cytokines such as IL-10.[17] In diabetic conditions, the macrophages phenotypic switch from pro-inflammatory (M1) to anti-inflammatory macrophages (M2) that play a role in IL-10 production could be delayed or fail to occur.[18] As a result, in patients with DM, there is excessive production of pro-inflammatory cytokines that can lead to tissue damage and delayed wound healing process.

In this study, it was revealed that 12% spirulina and 20% chitosan combination could be used to block the NF-κB pathway so the amount of IL-1β and TNF-α will decrease. The decreasing IL-1β and TNF-α level by 12% spirulina and 20% chitosan combination were mediated by C-phycocyanins contained in spirulina and N-acetyl-D-glucosamine oligomers (COS) in chitosan. Besides C-phycocyanin, spirulina also has other active compounds such as β-carotene. β-carotene and C-phycocyanin can block NF-κB activity and thus suppresses TNF-α, and iNOS expression. Besides, β-carotene also inhibits the expression of pro-inflammatory cytokines such as IL-1β and IL-6 in stimulated macrophages by suppressing their transcription.[19] As an antioxidant, β-carotene can inhibit ROS accumulation in intracellular. Consequently, oxidative stress that occurs due to ROS accumulation in the state of hyperglycemia could be reduced so that wound healing can occur faster.[20] Thus, these early studies showed that phycocyanin and β-carotene are significant contributors to the antioxidant and anti-inflammatory activities of Spirulina.[8] In the present study, 12% spirulina and 20% chitosan combination can

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only increase the amount of IL-10 on the 1st day after tooth extraction. Chitosan has various biological activities, including anti-inflammation and anti-oxidant. Besides, Chitosan is also able to accelerate the wound healing process by activating B lymphocytes and T lymphocytes, enhancing cellular and humoral immune functions and activating the tumour-killing activity of macrophages. The previous study suggests that chitosan is able to inhibit NF-κB, TNF-α, IL-1 β, IL-4 and IL-6 expression.[21]

According to the research by Neyrinck et al., IL-10 expression is not affected by spirulina supplementation.[22] This study also shows that several parameters involved in inflammatory such as IL-10 are significantly deregulated ($P < 0.05$) upon Spirulina treatment. The previous study shows that supplementation with Spirulina also inhibits TLR4 expression in the liver. Based on this study, spirulina given to the treatment might have no effects on M2 due to the inhibition in the TLR-4 expression. Several studies explain that TLRs stimulation results in the IL-10 gene transcriptional activation, thereby increasing the IL-10 protein production and secretion. TLRs will activate the MAPK pathways, which is essential for IL-10 production control in macrophages.[23] The previous study showed that phycocyanin in this substance has a promising anti-inflammation effect. Still, this effect is more connected to the anti-oxidant activities and is not connected to IL-10 improvement. Spirulina can increase the activities of the antioxidant enzymes like catalyze antioxidant enzyme (CAT) and superoxide dismutase (SOD) with a significant reduction on lipid peroxidation markers. In addition, it can also inhibit the inflammatory marker production such as prostaglandin (PGE2), TNF- α, IL-1 β, and IL-6.[24] However, the chitin content on chitosan can reduce iNOS and increase IL-10 production through activation of the signal transducers and activator of transcription-3 (STAT3) signalling pathway, this pathway activation is associated with NO inhibition and IL-10 production. This is in accordance with earlier studies that explain that IL-10 will interact with its high-affinity receptor IL-10R1 and low-affinity receptor IL-10R2 and lead to STAT signalling pathway activation that has a role in IL-10 signalling.[25] In this study, there is a possibility that the M2 cells target which has a role in producing anti-inflammatory cytokines do not get the most significant effect either from the spirulina or the chitosan, even though both of these materials have anti-inflammation and anti-oxidant activities. This shows that chitosan can increase the amount of IL-10, while spirulina will decrease the production of the pro-inflammatory cytokines such as TNF- α and IL-1β.

This study had a limitation on the number of rats enrolled. This study included 36 males rat, the higher number of samples will show more effectiveness of a combination of 12% spirulina and 20% chitosan for the amount of pro-inflammatory and anti-inflammatory cytokines. In addition, this study only observed the expression of cytokines in DM patient so the number of cytokines cannot be compared with normal conditions with the same treatment. Furthermore, in this study, the amount of IL-1β, TNF- α, and IL-10 expressions were also only observed by one observer, it could make the results of the cytokine count was strongly influenced by the observer's subjectivity assessment and could produce an inaccurate assessment.

DISCUSSION

In conclusion, 12% spirulina and 20% chitosan combination can decrease the amount of pro-inflammatory cytokines such as TNF- α and IL-1β significantly because both of these materials are anti-inflammatory and anti-oxidant through the mechanism of blockading the NF-κB. Furthermore, even though 12% spirulina and 20% chitosan combination do not give any significant effect on IL-10, there is still a significant improvement of IL-10 on treatment groups at day one and IL-10 improvement in general. This study showed that 12% spirulina and 20% chitosan combination still gives beneficial effect in increasing the number of anti-inflammatory cytokines in people with DM.

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ETHICAL CLEARANCE

This study was granted ethical approval no. 225/HREC.FODM/V/2019. This study was done at the Biochemistry Laboratory of Faculty of Medicine, Universitas Airlangga; Faculty of Pharmacy, Universitas Airlangga; Research Center of Faculty of Dental Medicine, Universitas Airlangga and Biochemistry Laboratory of Faculty of Medicine, Universitas Brawijaya.

CONFLICT OF INTERESTS

The authors declare that there were no conflicts of interest in the process of this study.

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AUTHOR CONTRIBUTION

All of authors are equally contributed to the study from the study framework, data gathering, data analysis, until reporting the result of study.

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