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Research Report

The effect of chitosan gel concentration on neutrophyl and macrophage in gingival ulcer of Sprague Dawley rat

Tasya Adistya,[1] Fajar Kumalasari,[1] Anne Handrini Dewi[2] and **Mayu Winnie Rachmawati[2]**
[1]Faculty of Dentistry, Universitas Gadjah Mada
[2]Departement of Biomedical Dentistry, Faculty of Dentistry, Universitas Gadjah Mada
Yogyakarta – Indonesia

ABSTRACT

Bacground: *Chitosan is polysacharide that extracted from crustaceae, widely used as a wound healing agent. It accelerates the polimorphonuclear cells infitration and increase the macrophage migration.* ***Purpose:*** *The aims of this study was to determine the effect of chitosan gel concentration on neutrophyl and macrophage in gingival ulcer healing process of Sprague Dawley rats.* ***Methods:*** *Twenty subjects were divided into treatment groups, A, B and C which was given 1%, 2% and 3% chitosan gel respectively and group D as control group. The ulcer was made by applicating the 2 x 2 mm^2 Whatmann number 1 filter paper which had been soaked into the 98% acetic acid for 5 minutes on the gingival surface below the interdental of the lower incisivus of the rats for 40 seconds. One drop chitosan gel was applicated on the ulcer, twice a day for three days. The subjects were sacrificed and its gingival tissue was taken for histologically processed and stained with hematoxylin eosin.* ***Results:*** *The one way ANOVA test showed that significant difference neutrophyl and macrophage among all of group ($p<0.05$). The Pearson correlation showed that there was a strong correlation (0,979) between chitosan concentration and macrophage density. Chitosan gel with 1%, 2%, and 3% concentration influenced significantly to neutrophyl and macrophage density. The higher concentration of chitosan gel the power of neutrophyl number and the higher of macrophage number.* ***Conclusion:*** *These result indicated that the chitosan gel influence both of neutrofil and macrophage in gingival ulcer healing process and chitosan gel 3% has a better effect than 1% and 2% concentration.*

Key words: *Chitosan gel, wound healing, gingival ulcer, neutrophyl, macrophage*

ABSTRAK

Latar belakang: *Kitosan, polisakarida hasil ekstraksi dari golongan krustasea, dikenal sebagai agen pemacu penyembuhan luka. Kitosan dapat memacu infiltrasi sel-sel polimorfonuklear dan mempercepat migrasi sel makrofag.* ***Tujuan:*** *Penelitian ini bertujuan untuk meneliti efek gel kitosan dalam meningkatkan jumlah neutrofil dan makrofag selama proses penyembuhan luka buatan pada gingiva mulut tikus Sprague Dawley.* ***Metode:*** *Duapuluh subyek dibagi atas 4 grup. Grup A dioles dengan 1% gel kitosan, grup B 2% dan grup C 3%, sedangkan grup D sebagai kontrol tidak mendapatkan perlakuan apapun. Ulkus dibuat dengan cara mengaplikasikan kertas saring Whatmann nomer 1 ukuran 2 x 2 mm^2 yang telah dibasahi dengan 98% asam asetat selama 5 menit dan diletakkan pada gingiva di bawah interdental gigi anterior mandibula selama 40 detik. Satu tetes gel kitosan diaplikasikan 2 kali sehari selama 3 hari. Tikus dikorbankan pada hari ketiga dan jaringan gingivanya diambil untuk dibuat preparat histologi dengan pewarnaan HE. Jumlah neutrofil dan makrofag dianalisa dengan ANOVA satu jalur.* ***Hasil:*** *ANOVA satu jalur menunjukkan adanya perbedaan bermakna pada neutrofil maupun makrofag antar grup ($p<0,05$). Korelasi Pearson menunjukkan ada hubungan positif antara konsentrasi gel kitosan dengan jumlah makrofag. Gel kitosan dengan konsentrasi 1, 2 dan 3% secara bermakna mampu menurunkan jumlah neutrofil dan meningkatkan jumlah makrofag pada hari ke 3 dibandingkan kelompok kontrol. Semakin tinggi konsentrasi gel kitosan maka jumlah neutrofil semakin menurun sedangkan jumlah makrofag semakin meningkat. Hal ini membuktikan adanya sifat antimikrobial dari gel*

kitosan. ***Simpulan:*** *Gel kitosan terbukti mampu menurunkan neutrofil dan meningkatkan makrofag pada proses penyembuhan ulkus mulut. Konsentrasi gel kitosan 3% mempunyai efek lebih baik dibandingkan dengan konsentrasi 1% dan 2%.*

Kata kunci: *Gel kitosan, penyembuan luka, ulkus gingiva, neutrofil, makrofag*

Correspondence: Anne Handrini Dewi, Bagian Biomedika Kedokteran Gigi, Fakultas Kedokteran Gigi, Universitas Gadjah Mada. Jl. Sekip Utara No 1 Bulaksumur, Sleman, Jogjakarta 55281, Indonesia. Email: anne_ikgd@ugm.ac.id

INTRODUCTION

The wound healing process are divided into three phases: inflammatory, proliferation, and scar maturation, that set in within minutes after skin injury to several months finally enters the maturation phase. The first inflammatory cell are leukocytes, namely neutrophils, monocytes and lymphocytes are recruited at different steps.[1] Neutrophils are important members of the innate immune and they play a role in the inflammation process. Neutrophils contain defisins, proteins that have a broad range of antibiotic activity for bacteria and fungi.[2] Neutrophils migrate across endothelial from local blood vessels and initiate wound healing by releasing early-response proinflammatory cytokines, such as TNF-α, IL-1-α and IL-1-β. The other leukocytes cell is monocytes that migrate from blood into tissue and differentiate into macrophages. Activating macrophages release many growth factors and cytokines including platelet-derived growth factor (PDGF), transforming growth factor beta 1 (TGF)-β1, as well as TNF-α and IL-1, which upregulate the ECM production. Neutrophils and macrophages are active in phagocytosis some bacteria, fungi and dispose of dead matter.[1-3]

Chitosan is natural polymers composed by randomly β-(1-4)-linked D-glucosamine (deacetylated unit) and N-aceytl-D-glucosamine (acetylated unit).[4] Chitosan is soluble only at aqueous acidic solution with pH<6.5. Negative characteristic of chitosan is its poor solubility at physiological pH.[5] Chitin and chitosan have been widely studied in both engineering and medicine due to its low cost, large scale availability, high biocompatibility, biodegradability and wound healing properties.[6,7] Several studies report the effects of chitin and chitosan on tissue reaction involved in wound healing.[3,4,8,9] They accelerate infiltration of inflammatory cells, stimulate angiogenesis, induce the rapid formation of vascular granulating tissue, the disappearance of purulence and they promote skin regeneration with minimal scar formation. They also have candidacidal and bactericidal activity.[4]

In the oral application, both chitin and chitosan, can be used in tooth paste, mouth washes, chewing gum, freshen the breath and prevent the formation of plaque and caries.[10] Due to its biocompatibility and biodegradability, chitosan have a great potential to be a drug delivery system (DDS).[11] Among the various approaches to modifying DDS, aiming to increase the ability to remain attached to mucous membranes such in oral mucous with complex condition including salivary system and pH changes. This study tried to develop chitosan property as the oral ulcer healing substance. The ideal treatment for oral ulcer should improve stimulating mucosal cell growth and removing bacterial cells that otherwise retard the healing process. The aim of this study was to determine the effect of chitosan gel concentration on the neutrophyl and macrophage in gingival ulcer healing process of Sprague Dawley rats.

MATERIALS AND METHODS

Chitosan powder 95% degree of deasetilation (food grade) and 1% acetic acid solution were prepared as materials for chitosan gel. Chitosan powder that has been weighed was put into measuring glass then were added 1% acetic acid solution till 100 ml in volume, stirred well. The comparison between chitosan powder and 1% acetic acid solution within chitosan gel 1%, 2% and 3% concentration was done.

This research was done an experimental laboratory and was done in histology laboratorium, Faculty of Dentistry, Universitas Gadjah Mada. The use of animal protocol was approved by the Bio-Ethics Committee of The Faculty of Dentistry, Universitas Gadjah Mada. National guidelines for the care and use of laboratory animals were applied during the study for 1 month. The animals were housed in cage located at the Integrated Research and Testing Laboratory, Universitas Gadjah Mada. Twenty healthy male Sprague-Dawley rats, 2-2,5 month old, weighing 200-250 gr were used for this study. Four groups randomly A, B, C which applicated with 1%, 2%, 3% chitosan gel respectively, and group D as negative control have prepared well.

Surgery was performed under general anesthesia by intramuscular injection of ketamine 50 mg/kg body weight in combination with xylasin 0.5-1.1 mg/kg body weight on left upper leg intramusculary. To reduce the risk of perioperative infection, the rats were treated with antibiotics, interflox-100 (Interchemix, Holland) at 10 ml/20-40 kg intramusculary during 3 days after injury created. Whatmann filter paper no. 1 with size 2 x 2 mm soaked into 98% acetic acid solution for 5 minutes.[12] The lower lip was retracted and the Whatmann filter paper was applicated to labial gingival below the interdental both of anterior mandibular for 40 seconds with no pressure.

Gingival ulcer was formed two days after the acetic acid application.

One drop chitosan gel applicated for the treatment groups twice a day, in the morning (6 a.m) and afternoon (4 p.m) for 3 days. When the gel was applicated, the lower lip was retracted and kept retracted for about 1 minute until the gel penetrated well into gingiva tissue. On the 3rd day all of the rats were sacrificed and its gingival tissue were taken for histologically processed and stained with Hematoxillin Eosin. The data was collected by counting the amount of neutrophyl and macrophage under the light microscope. The result was ratio data and were statistically analyzed for neutrophyl by one way ANOVA and LSD, meanwhile for the macrophage by one way ANOVA, LSD, and Pearson Correlations.

RESULTS

Microscopic analysis on the rats gingival ulcers slides showed a high infiltration of neutrophyl PMN as the bluish purple cells with 2-5 loby nucleus and red granuled cytoplasm. Macrophage was round to oval in shape and has a nonflat border. The excentric nucleus of macrophage was smaller than its on fibroblast and stained darker because of the phagocytosed particles. Macrophage's nucleus is ovoid in shape and has a fold on a side like kidney-shaped.[13,14]

Table 1 for the group C (3% chitosan gel) showed that the lowest of neutrophyl but the highest of macrophage. The chitosan gel with concentration 1%, 2% and 3% for three days aplication influenced the amount of neutrophyl and macrophage on the ulcer healing of rats' gingiva. The result of the one way ANOVA showed that there was a difference among groups 0.001 ($p<0.05$) for neutrophyl and macrophage. Meanwhile, the Pearson corellation test showed that there is positive and strong correlation (0.979) between the concentration of chitosan gel and the amount of macrophage (Figure 1).

The result of the LSD analysis showed that there was a significant difference ($p<0.005$) for both of neutrophyl and macrophage among each group (Table 2). From the data analysis above, it showed that chitosan gel with 1%, 2% and 3% concentration influence the amount of neutrophyl and macrophage. The higher concentration of chitosan the lower number of neutrophyl and the higher number of macrophage Histological examination result was show in Figure 2 at days 3 after application. Histological examination result was shown in Figure 2.

DISCUSSION

Chitosan is a linear polysaccaride composed of randomly distributed of glucosamine and N-acetylglucosamine units linked by 1-4 glucosidic bonds. It is obtained by N-deacetylation of chitin, which is the second most naturally occuring biopolymer after cellulose.[13] Chitosan is distinct from other polysaccarides due to the presence of nitrogen in its molecular structure that makes to be cationic charge and its capacity to form polyelectrolyte complexes. This cationic side allows it become water soluble after the formation with kind of carboxylate salts (formate, acetate, lactate, malate, citrate, glyoxylate, pyruvat, glycolate and ascorbate).[14]

The wound healing process is complex interaction among cells, extracellular matrix component and signaling pathway between them. To achieve optimal good healing, maintaining a moist wound healing environment, preventing and managing infection are very important. The local delivery of therapeutics to the mouth can be used to treat a number of diseases, such as periodontal disease, stomatitis, fungal or viral infection and oral ulcer like oral mucositis. Ulcer is a kind of wound that mostly happened because of many agents, such as physical trauma, chemical substance, allergy, infection, neoplasma, systemic disease and imunity disorder.[15] In oral cavity due to many bacteria and masticatoria activity, it will increase a risk of infection. In addition, drug administration through the oral mucose must consider to flushing action of saliva and should be formulated to prolong retention of the drug in the oral cavity.

The use of chitosan in this experiment was in gel form. Shemer *et al*.,[16] used a 'gel like' pharmaceutical to treat aphthous ulcers and it is applied directly on the ulcer. The application of chitosan gel may effectively interact with and protect the wound, ensuring a good, moist healing environment.[17] Bioadhesive polymers have been utilized in a gel forms to prolong the retention on oral mucose. Due to

Table 1. Average and standar deviation of neutrophyl and macrophage

Group	Average ± SD	
	Neutrophyl	Macrophage
A (1%)	500.361 ± 11.312	176.39 ± 18.8
B (2%)	284.217 ± 3.081	278.25 ± 17.0
C (3%)	139.759 ± 5.418	401.56 ± 19.6
D (Control)	656.807 ± 21.529	119.55 ± 5.00

Table 2. The LSD analysis of the neutrophyl and macrophage

Group	Significantly
A-B	0.001*
A-C	0.001*
A-D	0.001*
B-C	0.001*
B-D	0.001*
C-D	0.001*

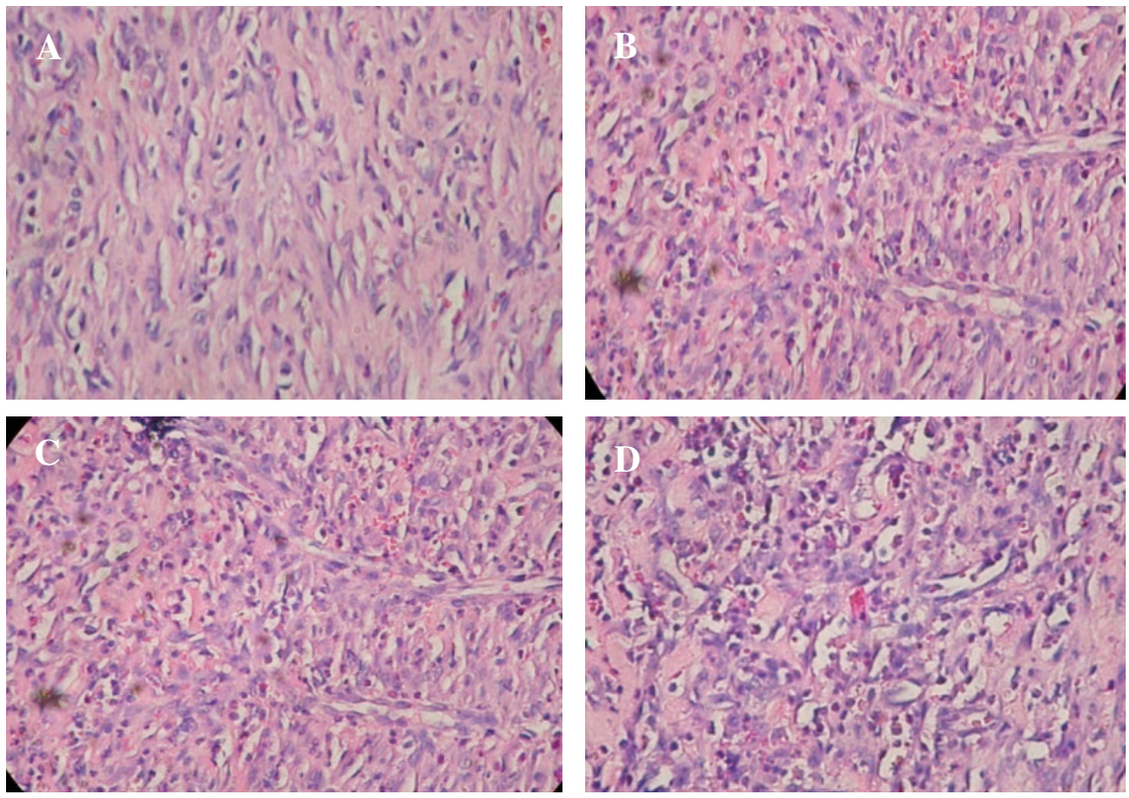

Figure 2. Histological examination of group A, B, C and D, showed infiltration of neutrophyl and macrophage on the third day with 400x magnification.

muco adhesive properties, chitosan have been recognized as excellent candidates for oral delivery system.[14,18] Sinha *et al*.[17] reported that the chitosan membrane was found to adhere uniformly over artificial created wound on the dorsum of the mice and also adsorbed the exudates from the wound surface.

Based on Table 1, neutrophyl cells on group C (chitosan 3%) after application twice a day for 3 days, has the lowest compared with group A (chitosan 1%), B (chitosan 2%) and control group. The inflammation process set in within minutes after injury. On the 3rd day, amount of neutrophyl cells will decreased gradually by physiologycal process and the neutrophyl cells on chitosan group were lower than control group. The finding was similar to Elassad[19] that reported when chitosan was implanted in the rat's peritoneal cavity, it induced a significant celluler response with recruitment of inflammatory cells as well as the other immune cells to the wound site at 4 hours after implantation. The percentage of PMN was estimated to be 28.4% for chitosan group, greater than 18.5% for control group by Gelfoam®. This indicated that chitosan accelerate PMN cell to wound site better than control group. At 3 days after chitosan application, the result showed the decreased of neutrophyl. We suggest that chitosan can accelerate elimination debris substance and any bacteria that is done by neutrophyl.

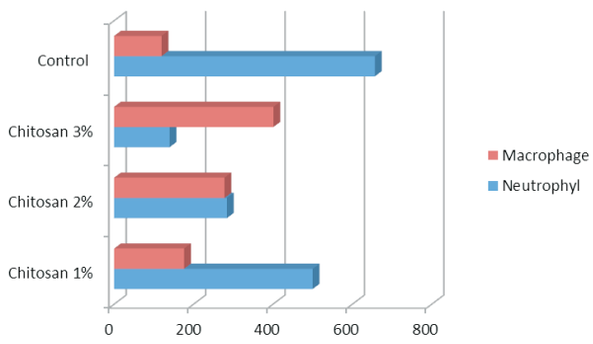

Figure 1. The average of neutrophyl and macrophage after 3 days aplication of chitosan gel.

Chitosan and chitin have been observed to accelerate wound healing by stimulate the migration of polymorphonuclear and mononuclear cells. It acts as chemo attraction and activates neutrophyl and macrophages to initiate the healing process.[3,4,20] Kojima *et al*.,[8] reported that at 2nd day, the inflammatory cells increased within implant of the chitin and chitosan groups but not in the control group. Chitosan was also reported has antimicrobial activity that against different groups of microorganism such as bacteria,

yeast, and fungi. There are two main mechanism have been suggested related of antimicrobial of chitosan. The first is interactions between both positive and negative charged molecules leads to increase the permiability of the outer and inner membranes of bacteria and resulting disruption of membrane integrity make releasing of the content of the cells.[21] It has been postulated that antimicrobial nature of chitosan is due to surface-surface interaction between the biopolymer chains and microbial cell walls.[22] The second mechanism suggest that chitosan may inhibit microbial growth by acting as chelating agent rendering metals, trace elements or essential nutrients that are needed for the organism to grow at the normal rate.[21]

Due to antimicrobial activity, chitosan can enhance elimination activity for some bacterial in the ulcer area. This action can reduce a number of neutrophyl since elimination of injurious agent on the ulcer tissue is done by neutrophyl phagocytosis. Increasing the content of the chitosan would enhance the anti microbial capacity.[23] Chitosan molecules was reported promoted migration of the inflammatory cells which were capable of the production and secretion of large proinflammatory product and growth factor at early phase of healing.[3] Chitosan stimulates interleukin 8 (IL-8) production which chemoattracs and recruits neutrophyls to the wound.[19]

This result, at 3 days, the macrophages cell showed the highest number by chitosan 3%. Macrophage plays an important role in the wound healing process, that is phagocyting the debris[4,23] and stimulates the secretion of growth factors, cytokines, and inflammatory mediators that is induce the activation of other cells like endotellium cells, epitelium cells and fibroblasts.[3,4] The growth factors produced by macrophage is TGF-α, bFGF, PDGF, HB-EGF and VEGF that can accelerate the cell proliferation and the synthesis the extracellular matrix. Lack of macrophage on the wound tissue will make the cleaning of the wound less optimal, delayed fibroblast proliferation, inadequate angiogenesis and bad fibrosis.[1,3] Activated macrophages are important in host immune defenses, but their uncontrolled activation can lead to septic shock and death.[4]

Chitosan accelerates the migration of PMN and induces macrophages,[4] so the group C (3% chitosan) showed the highest of macrophage than the other groups and Pearson correlation analysis showed that there was a positive correlation between the level of chitosan gel concentration and the increase of macrophage. Normally, the proliferation phase proceed over the next 5-14 days to initial process for repair both epidermal and dermal layer. Macrophages, fibroblast and vascular tissue together enter to the wound and begin to make the formation of granulation tissue that further to re-epithelialization process.[3] Kojima *et al.*[8] reported that many polykaryocytes from the fusion of macrophages were observed in implant with chitin at 7 days compared to the control group. Chitin at higher concentration stimulate platelets and macrophages, resulting in the release of PDGF and TGF-β but the phenomenon suggests influence locally only, not systematically.

In addition, based on their study, Mori *et al.*,[4] suggest that chitosan was found to induce macrophage apoptosis. It took 6 hr to indice apoptosis in 50% of the macrophages in cell culture. The gradual apotosis needed to control wound healing acceleration and inflamation. Chitosan activates macrophages for tumoricidal activity and for the production of interleukin-1. Oligochitosan had an in vitro stimulatory effect on the release of tumor necrosis factor-α and interleukin-1β.[3] Besides, chitosan has also in vivo stimulatory effect on both nitric oxide production, chemiotaxis, and modulates the peroxide production. Chitin or chitosan oligomers generated by chemo-enzymatic degradation in the wound environment, exert significant biochemical effects, migration of the mouse peritoneal macrophages was enhanced significantlyby chitin/chitosan oligomers.[4]

In conclusion, chitosan is a biomaterial already used in various medical devices. As the oral ulcer healing substance, it were prepared using acetic acid to make a gel solution with 1%, 2% and 3% concentration. All concentration group influence to decrease the amount of neutrophyl and increase the amount of macrophage on the Rats' gingival ulcer healing in three days after injury. The lowest of neutrophyl are founded in a highest concentration of chitosan. This is related to antimicrobial properties of chitosan. Interaction between anionic groups on the cell surface of microorganism and polycationic charge of chitosan decreased a number of bacteria. Meanwhile, this elimination process also are done by antimicrobial activity of chitosan, so that amount of neutrophyl cells on the ulcer area for treatment groups were lower than control group.

The result showed that by 3% concentration, whether the lowest of neutrophyl, but the macrophage were the highest. Chitosan can stimulate the chemotatic process of the macrophages due to its N-acetyl glucosamin group. At higher concentration it stimulates platelets and macrophages, resulting in the release of PDGF and TGF-β. This study suggested that chitosan gel with 3% concentration has a better effect than 1% and 2% concentration to enhance gingival ulcer healing in the rat. Chitosan can accelerate wound healing related to amount neutrophyls cells and macrophages at days 3 after application and have a good adhesive properties on oral mucous of Sprague Dawley rats. Further studies to develop chitosan as drug delivery system are needed for antibiotic or anti inflammation drug in oral application within membran shape to get a long contact with oral mucous. Since solubility of chitosan only in acidic condition, it is very important to consider physiologic pH in oral environment.

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