Dental Journal
Majalah Kedokteran Gigi
Volume 47, Number 1, March 2014

Research Report

Spirulina chitosan gel induction on healing process of Cavia cobaya post extraction socket

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ABSTRACT

Background: *Prominent residual ridge is necessary to gain retention and stabilility for succesful prosthodontic treatment such as removable, fixed or implant. Spirulina is a natural substance that can help tissue healing and chitosan also a natural substance that reported to have the ability to help bone remodelling. The combination gel of spirulina and chitosan could be considered as an alternative material to maintain residual ridge height after tooth extraction.* ***Purpose:*** *The aim of study was to examine the effect of combination gel of Spirulina and chitosan on healing process of Cavia cobaya post tooth extraction socket by counting the amount of osteoclast, osteoblast and colagen as an indicator.* ***Methods:*** *Twenty eight cavia cobaya were divided into 4 groups. Insisive mandible extraction was done and the sockets were filled with 3% CMCNa for control groups, 3% spirulina chitosan 200 mg for group 1, 6% spirulina chitosan 200 mg for group 2, 12% spirulina chitosan 200 mg for group 3. After 30 days, histopathology examination was done by using microscope to count the amount of osteoclast, osteoblast and collagen.* ***Results:*** *Data was analyzed by using Anova and Tukey HSD. For osteoclast, there was no significant different between every groups, while for osteoblast and collagen there was significant different between groups. The results showed that induction of combination gel spirulina chitosan was able to accumulate collagen fiber and resulting faster wound healing.* ***Conclusion:*** *Combination 12% gel spirulina chitosan 200 mg could be used as an alternative material for better bone remodeling after tooth extraction.*

Key words: *Spirulina, chitosan, bone remodeling, tissue healing, cavia cobaya*

ABSTRAK

Latar belakang: *Residual ridge yang prominen sangat dibutuhkan untuk mendapatkan retensi dan stabilitas untuk menunjang keberhasilan perawatan di bidang prostodonsia seperti pada kasus removable, fixed atau implant. Tindakan pencabutan gigi dapat merusak jaringan periodontal, sementum dan tulang alveolar yang mengakibatkan resorbsi ridge yang besar. Spirulina telah terbukti mempunyai kemampuan untuk membantu penyembuhan tulang sedangkan kitosan mempunyai kemampuan untuk membantu proses pembentukan tulang. Kombinasi kedua bahan ini diharapkan dapat menjadi bahan alternatif untuk mempercepat proses penyembuhan luka dan pembentukan tulang.* ***Tujuan:*** *Penelitian ini bertujuan meneliti efek induksi kombinasi gel dari Spirulina dan kitosan terhadap proses penyembuhan soket pasca ekstraksi gigi Cavia cobaya dengan indikator jumlah osteoklas, osteoblas dan kolagen.* ***Metode:*** *Penelitian ini menggunakan 28 marmot yang dibagi menjadi 4 kelompok penelitian. Pencabutan dilakukan pada incisive rahang bawah kemudian soket pencabutan diisi dengan CMCNa 3% pada kelompok control; spirulina 3% chitosan 200 mg pada kelompok perlakuan 1; spirulina 6 % chitosan 200 mg pada kelompok perlakuan 2, dan spirulina 12% citosan 200 mg pada kelompok perlakuan 3. Pada hari ke 30 dilakukan pemeriksaan histopatologi menggunakan mikroskop untuk menghitung jumlah osteoblas, osteoklas dan kolagen.* ***Hasil:*** *Data dianalisis dengan Anova dan Tukey HSD. Jumlah osteoklas tidak berbeda secara signifikan antara setiap kelompok, sedangkan jumlah osteoblas dan kolagen terdapat perbedaan yang signifikan antara kelompok. Hasil penelitian menunjukkan bahwa*

induksi kombinasi gel spirulina chitosan mampu mengakumulasi serat kolagen dan menghasilkan penyembuhan luka lebih cepat. ***Simpulan:*** *Kombinasi gel spirulina 12% chitosan 200 mg dapat digunakan sebagai bahan alternatif untuk remodeling tulang yang lebih baik setelah pencabutan gigi.*

Kata kunci: *Spirulina, kitosan, penyembuhan luka, pembentukan tulang, cavia cobaya*

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INTRODUCTION

Tooth extraction is a procedure that cause damage to the part of the tooth socket which includes periodontal tissues such as gingival, cementum, periodontal ligament and alveolar bone. Inflammatory process, which is the main result of tooth extraction, always followed by bone remodeling and tissue repair. Healing process need sterile state and materials containing anti-inflammatory, anti-bacterial, anti-mycotic, insecticidal, antiseptic and anti-parasitic to accelerate this process.[1]

In general, healing process consists of three phases, inflammatory phase, proliferative phase and remodeling phase. Inflammatory phase begins immediately after tooth extraction until 3 to 5 days. Cardinal signs such as rubor, tumor, calor, dolor and fungsiolaesa always happen in this phase. Production of histamine, kinin and prostaglandin by leukocytes increase.[2] Proliferative phase lasts for 3 days to 3 weeks. In this phase the tooth sockets filled with inflammatory cells, fibroblasts, collagen matrix, and hyaluronic acid which serve for the formation of granulation tissue.[3] Remodeling phase is also called maturation phase. In this phase of osteoblast cells will aggregate the intercellular substance of bone that contains collagen to form new collagen fibers. Activity of osteoblasts and osteoclasts will change immature bone (woven bone) become mature bone (lamellar bone). The states of the bones become stronger so that osteoclasts can penetrate tissue and debris in the injured area followed by osteoblasts that will fill the gap between the new bones. This happened a few months or even years until alveolar bone become its original form.[4]

Currently pharmaceutical technology development has focused worldwide attention on the ingredients derived from nature because relatively safe compared to chemical drugs. Spirulina has many nutritional benefits to the human body, such as C-phycocyanin, B-carotenoids, vitamin E, zinc and many trace elements and other natural phytochemicals . One of the ingredients derived from nature that has been researched and proven as an anti- inflammatory and antioxidant in wound healing process is C-phycocyanin or blue substance.[5] Gel concentration of 12% spirulina most effectively to increase the number of fibroblast cells after tooth extraction guinea pig (Cavia cobaya).[6]

Chitosan, product of chitin derivatives with the formula N-acetyl-D Glucosamine, is a cationic polymer that has number of monomers around 2000-3000 monomeric, non-toxic and molecular weight about 800 kD. Chitosan is produced from chitin deacetylation under alkaline conditions. Chitin can be obtained from the shells of crustaceans, insects and other sources. This biopolymer has good character, biodegradable, biocompatible, antibacterial properties and safe for humans.[7,8] Chitosan has been used as a drug delivery system, orthopedic implants and periodontal wound healing management, and scaffolds for tissue regeneration. In the field of wound healing, chitosan proved to activate immune cells, inflammatory cells such as PMN, macrophages, fibroblasts and cells angioendotelial. Natural healing of chitosan derived from its ability to stimulate the production of fibroblasts by affecting fibroblast growth factor.[9] Ariani *et al*.,[10] in his research 2013 using chitosan 200 mg said that chitosan has a porosity structure and good retention to support proliferation osteoblast cell.

The aim of study was to examine the effect of combination gel of spirolina and chitosan on healiny process of Cavia cobaya post tooth extraction secret by counting the amount of osteodast, osteoblast and colagen as an indicator.

MATERIALS AND METHODS

This research was an experimental laboratory by using the draft post-test only control group design. Experimental animals used in this study were Cavia cobaya, 2-3 months old, male, average body weight of 300 grams. Total of 28 cavia cobaya divided into 4 treatment groups, each group has consist of 7 animals. Experimental animals maintained for 3 days to adapt in the cages measuring 60 cm x 65 cm x 80 cm (7 animals per cage) and placed in the room light enough to avoid moisture, away from the noise and not exposed to direct sunlight. The food provided is corn and fresh carrots.

Spirulina was in powder form produced by Wellness USA. Chitosan was in powder derived from the shells of shrimp produced by Soetomo Hospital Tissue Bank. Combination gel was the result of mixing spirulina powder and chitosan powder with base gel CMC Na 3% to produce stable gel consistency. Base gel 3% Na CMC does not affect the gel function, viscosity so it's as treatment to control group.

Chitosan that used for each treatment group is 200 grams. The combination of gel for this study were as follows, treatment group 1, 3% of spirulina from 300 mg spirulina, 9.5 g of CMC Na 3% and 200 mg chitosan,

treatment group 2, 6% of spirulina from 600 mg spirulina, 9.2 g of CMC Na 3% and 200 mg chitosan, treatment group 3, 12% of spirulina from 1200 mg spirulina, 8.6 g of CMC Na 3% and 200 mg chitosan.

Treatment was done by extracted left mandibular incisors Cavia cobaya using modification of the needle holder under anesthesia 10% inhalation. After extraction, socket was filled with combination gel using 0.1 cc syringe then closed by former revocation stitched using silk threads 3/0. Animal was feeding as usual until day 30 then Cavia cobaya was executed with 10% ether anesthesia to remove the mandible then performed decalcification with 2.5% nitric acid for 2 days. After mandibular bone tissue becomes soft, cutting incisor socket area was done in rectangular shaped cuts in the sagittal direction. Results of the pieces was immersed in 10% buffered formalin for 24 hours. Further processed for making preparat histopathological anatomy (HPA) with haematocylin eosin staining (HE).

Observations were made on preparat HPA and divided into three random visual fields by counting technique using a beta counter. Region that will be calculated was the socket that filled with combination gel. Counting the number of osteoclasts, osteoblasts and collagen was conducted with binocular microscope lens with magnification 1000x connected directly to the computer.

Collagen (Figure 1A) the most fiber in the human body, was observed shaped thick, sinuous, consisting of inelastic collagen protein (white fibers), pink color was obtained as the staining was using HE. Osteoblast cells (Figure 1B) was observed cuboidal or cylindrical-shaped short, have a cell nucleus, cytoplasm red and blue in microscopically. Osteoclasts (Figure 1C) was observed multinucleus form giant cells, round or oval, blue and red cytoplasmic surface located on the side of the resorbed bone slight rough.

Tabel 1. Multiple comparison test Tukey LSD osteoblast, osteoclast and collagen

		Control	Group 1	Group 2	Group 3
Osteoclast	Control	-	-	-	-
	Group 1	-	-	*	*
	Group 2	-	*	-	-
	Group 3	-	*	-	-
Osteoblast	Control	-	*	-	-
	Group 1	*	-	*	*
	Group 2	-	*	-	-
	Group 3	-	*	-	-
Collagen	Control	-	-	-	*
	Group 1	-	-	-	-
	Group 2	-	-	-	-
	Group 3	*	-	-	-

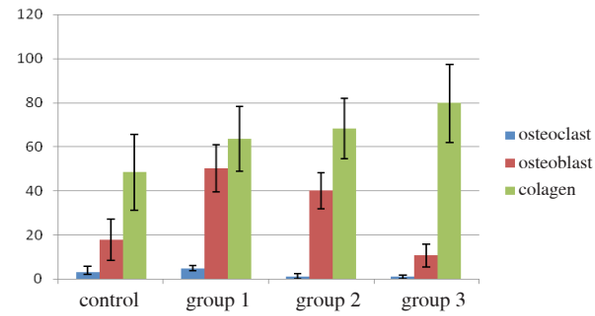

Figure 2. Mean and standard deviation of osteoblast, osteoclast and collagen of Cavia cobaya socket after treatment with spirulina chitosan gel.

RESULTS

The results of counting the number of osteoclasts, osteoblasts and collagen can be seen in the Figure 2. The results of osteoclasts numbers was increased in treatment group 1 compared to the control group, but in treatment groups 2 and 3 osteoclasts decreased compared with the control group. The results of osteoblasts numbers appears that the highest number of osteoblasts were in treatment group 1, while the lowest number of osteoblasts were found in treatment group 3. The results of counting the numbers of collagen appears that cells in the treatment group 1, 2 and 3 increased compared to the control group.

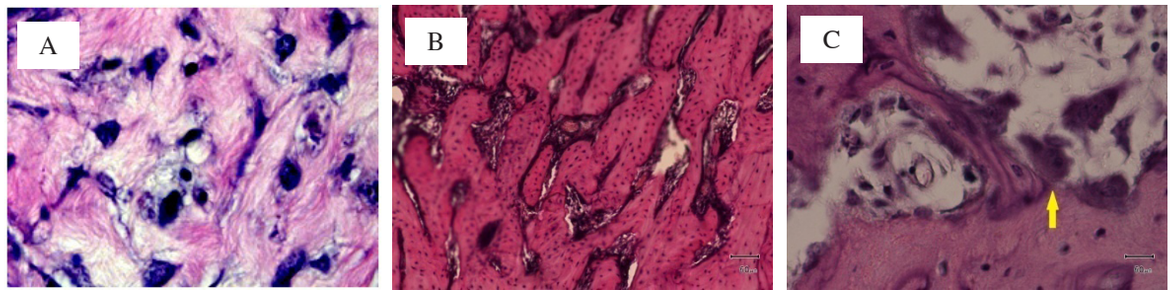

Figure 1. Histological examination with HE staining (A) collagen, (B) osteoblast, and (C) osteoclast.

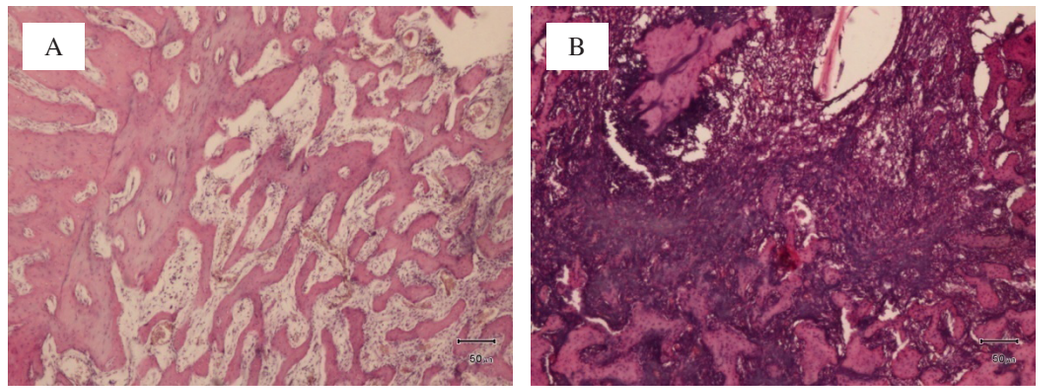

Figure 3. Histological section of healing socket 30 days after ekstraction with HE staining. A) minimal granulation tissue, newly formation bone can be seen, B) granulation tissue with minimal bone formation.

The results of the Kolmogorov and Smirnov test statistic Lavene showed that all data were normally distributed and homogeneous then proceed with the ANOVA test. To determine differences in the number of osteoclasts, osteoblasts and collagen in each treatment group and control group Least Squares Different test was used (Table 1).

DISCUSSION

Cavia cobaya was chosen as experimental animals as its metabolic systems anatomically and physiologically similar to humans. Lower incisor was chosen because the tooth sockets was deeper and larger than the other teeth, always calcified and continuously erupting so that the tooth crown can be as high as elongated molars. Lower incisor shaped like a segment of scissors or a cutting tool that resembles a pair of scissors. It is easier for researchers to incorporate material combination of spirulina and chitosan gel in a tooth socket after a tooth extraction so that the socket wound healing process can be observed.[11]

This research used male cavia cobaya because its hormonal system more stable compare to female. Hormonal systems in female cavia cobaya will affect growth hormones production such as epidermal growth factor (EGF), which is a hormone compound derived from blood platelets in addition to PDGF and TGF that have important role in wound healing.

Counting the number of osteoblast, osteoclast and collagen was done on third cervical tooth socket as it was the healing center. Based on Table 1, treatment group 1 (combination of spirulina 3% and 200 mg of chitosan) numbers of osteoclasts was increased compared to the control group, where as in treatment group 2 (combination spirulina 6% and chitosan 200 mg) and treatment group 3 (combination spirulina 12% and 200 mg of chitosan) was decreased. Considering only with these results, there is unmatch between existing theories and this research which actually increased the number of osteoclasts. In the process of bone remodeling there is a close relationship between osteoclasts and osteoblasts because these two cells cooperate together in bone remodeling process. Therefore, its important to consider the number of osteoblasts in the number of osteoclasts. It turns out that the number of osteoblasts directly proportional to the number of osteoclasts in this study. It was proven that bone remodeling happened because of the balance amount of osteoblasts and osteoclasts.

The results of this research showed that osteoblast cells increased by induction of a combination spirulina and chitosan gel. Osteoblast in treatment group 1 increased compared with the control group, but in treatment group 2 osteoblast significantly decreased compared with the treatment group 1. In treatment group 3 osteoblast also decreased although not significantly different from treatment group 2. This happened because observation was done in day 30^{th} and the combination gel can regenerate bone remodeling. Therefore osteoblast numbers become less because its change into bone matrix and this was proven by observation in the microscope that in treatment group 2 was seen less granulation tissue compared with treatment group 1. Observations in the treatment groups 3 showed osteoblasts was getting more difficult to find as well as the formation of islands of bone growth or spicules are fused and form a branching to make nets bone hence granulation tissue at this stage has not looked. This can be seen from histological examination in figure 3A and 3B.

The result was clearly visible on the socket preparations that were observed under a microscope. In the control group visible scars of tooth extraction socket area was still a lot of granulation tissue and little bone was formed. In the treatment group 1 visible granulation tissue began to decrease replaced by bone matrix so that the number of osteoblasts and osteoclasts increased. This indicate that bone remodeling process was going on. In the treatment group 2 appeared to have less granulation tissue and bone

matrix which was quite a lot, in the 3 treatment groups were seen socket begins to fill with the bone matrix of the surrounding granulation tissue (Figure 2). This indicates that the treatment group 2 and 3 bone remodeling occurs faster than the control group and the treatment group 1.

The number of osteoclasts and osteoblasts were decreased in treatment groups 2 and 3 due to the phase formation of osteoclasts and osteoblasts peak had passed and many formed bone in the socket. According Miloro,[12] osteoclasts begin to resorb alveolar crest in the first week and the second week will be even greater resorption. While the number of osteoblasts peaked at 6-8th week.[13]

Amler *et al*., *cit*. Mezzomo *et al*.[14] stated that human alveolar bone can cured histologically without any drug, and after 4 weeks of tooth extraction will occur naturally in the process of osteoblastic bone tissue formation. In this study it appears that the islands have been formed bone growth (spicules) are fused and form a branching to create webs of the former bone in tooth extraction sockets within 4 weeks. This suggests that administration of a combination of spirulina and chitosan gel capable to speeding up the process of bone remodeling. The results of the study showed that the induction of combination gel spirulina 12% chitosan 200 mg showed significant results compared with the control group. This suggests that this combination gel able to accumulate collagen fiber and resulting faster wound healing.

In this study, collagen density was used as an indicator in wound healing because collagen plays an active role in the proliferation stage which starts up with the maturation phase. Collagen was first detected on 3rd day after injury and increased until 3rd week. Collagen fibers will continue to accumulate until 3 months. Specific function of collagen is to make the new tissue (connective tissue matrix) and the release of substrates by fibroblast cells will give a mark on macrophage cells and new blood vessels and fibroblasts as well as one unit in order to enter the area of the wound so that the process of granulation is formed.[15]

Spirulina has a high alkaline properties, about pH 9 to 11.[16] Chitosan has a pH of 6.2 to 7.[17] Mixing the two materials make the pH becomes slightly more alkaline. Slightly more alkaline atmosphere necessary for alkaline phosphatase activity that contributes to mineralization.[4] In the bone remodeling process, osteoblast has an important role. Osteoblasts are a major component in this process to synthesize new bone tissue resulting in the formation of the alveolar bone.[18]

Tooth extraction can lead to complications such as inflammation in the tooth socket. An inflammation can also occur in the healing phase socket, this happened because the first defense cells were activated such as macrophage. Macrophages are phagocytic cells that are produced in the spinal cord that plays an important role in inflammation, such as bacteria digest and remove the unwanted cell lysis or that have been damaged. These macrophages would trigger the secretion of proinflammatory cytokines such as tumor necrosis factor (TNF), interleukin 1 (IL-1) and interleukin 6 (IL-6) as a mediator of inflammation to amplify the immune response and increase in metabolic processes. Along with this, macrophages activate nuclear factor-kappaB (NFkB). NFkB transcription factor is a protein in macrophages which are activated as a result of a bacterial toxin. This process will lead to an increase in proinflammatory mediators such as TNF, IL-1,and IL-6. If NFkB increases, three proinflammatory cytokine genes will also increase, because TNF, IL-1 and IL-6 are interconnected to stimulate inflammation.[19]

According Aranaz *et al*.,[9] Dai *et al*.,[20] and Pinto,[21] chitosan is able to improve the function of inflammatory cells such as polymorphonuclear leukocytes (PMN), macrophages, fibroblasts and osteoblasts to help bone formation. Spirulina contains phycocyanin as an anti-inflammatory which will suppress excessive inflammatory reaction after tooth extraction. Phycocyanin and carotenoids work on macrophages via toll like receptor (TLR) by suppressing the activity and inhibit the translocation of NFkB, which will reduce the excessive expression of proinflammatory cytokines such as TNF σ, IL-1 (interleukin-1) and IL-6. Osteoblasts express osteoprotegerin (OPG), which serves as the receptor binding of RANKL (receptor activator of NF-kappaB ligand) that blocks RANKL binds to RANK. OPG binds to RANKL, thus preventing the activation of osteoclasts. Decreasing the amount of production of proinflammatory cytokines (TNF-α, IL-1 and IL - 6) led to decreased RANKL is also expressed. Increasing OPG and RANKL will lead to a decreased in active osteoclasts. Decreased osteoclast resorption will reduce during bone remodeling.[22-24]

The study suggested that combination gel 12% spirulina chitosan 200 mg could be used as an alternative material for better bone remodeling after tooth extraction.

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