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Research Report

Topical application of snail mucin gel enhances the number of osteoblasts in periodontitis rat model

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ABSTRACT

Background: *Repair of bone damage represents a fundamental issue in the treatment of periodontitis. The important indicator employed to monitor the bone damage repair process is the number of osteoblast cells. Achatina Fulica snail mucin (SM) contains glycosaminoglycans which have the potential to increase their number. However, the use of SM in dentistry remains limited.* ***Purpose:*** *To determine and prove the effect of SM gel in increasing the number of osteoblasts in rat models suffering from periodontitis.* ***Methods:*** *This study used 36 rat models divided into three groups, namely; a treatment group (T: 20% snail mucin gel, n = 12), a positive-control group (P: hyaluronic acid gel, n = 12) and a negative-control group (N: CMC-Na gel, n = 12). 0.2 ml of all material was applied to a pocket by means of a tuberculin syringe once a day for 14 days. Histologic observations using Haematoxylin-Eosin staining were carried out on days 3, 5, 7 and 14. Data was analyzed by two-way ANOVA followed by a post-hoc LSD.* ***Results:*** *A significant difference existed between the number of osteoblasts in the test groups. The highest number of osteoblasts observed was consistently that in the treatment group.* ***Conclusion:*** *The application of 20% snail mucin gel was effective in enhancing the number of osteoblasts in rats suffering from periodontitis.*

Keywords: *bone repair; osteoblast; periodontitis; snail mucin (Achatina fulica)*

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INTRODUCTION

Periodontal disease constitutes an inflammation affecting the tissues surrounding the teeth, i.e. the gingiva, periodontal ligament, cementum and alveolar bone.[1] Research conducted in 497 districts/cities across Indonesia has indicated that the prevalence of periodontal disease is one of 95.21%.[2] This situation pertains as the result of persistent infection and inflammatory responses to periodontal pathogens which subsequently cause progressive changes in and damage to the gingiva, periodontal ligaments and alveolar bone around the teethresulting in tooth mobility.[1,3]

Bone healing represents an important objective of the treatment of periodontal disease. The healing process in alveolar bone consists of three phases, namely: inflammation, proliferation and bone remodeling. Osteoblasts, as one indicator of bone healing, play a role in the remodeling phase by secreting osteoid (bone matrix). At the outset of bone formation, osteoblasts synthesize basic substances and collagen which undergo a polymerization process resulting in the formation of collagen and tissue fibers and, subsequently, osteoid which mineralizes to become bone.[4]

One common therapy in the treatment of periodontitis is the topical application of antimicrobial therapy and hyaluronic acid.[5,6] Hyaluronic acid (HA) can weaken the bonds of chronic inflammatory tissue cells with the result that they are readily released and replaced through the regeneration of healthy cells. It also produces antimicrobial effects on *Aggregatibacter actinomycetemcomitans, Provotela oris, Porphyromonas gingivalis and Staphylococcus aureus*.[7]

The availability of snails in Indonesia has long promoted the use of snail mucin as a traditional medicine by groups of

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herbal sellers from Solo to manage tooth-related infections by dripping it onto perforated teeth. Snail mucin contains glycosaminoglycans consisting of heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate, keratan sulfate and hyaluronic acid that play many important roles within biological systems.[8] During bone regeneration, HA induces the stages of osteogenesis as a continuous extracellular matrix component.[9] The interaction between heparane sulfate and bone morphogenetic protein (BMP) antagonists will inhibit the activity of inhibitors and potentiate BMP activity during bone healing.[10] Heparin functions in an almost identical manner to heparane sulfate in that it affects the activity of BMP.[11] Interestingly, snail mucin also contains antibacterial achasin. The study described below was undertaken to determine and prove the effect of snail mucin gel in increasing the number of osteoblasts in rat models suffering from periodontitis.

MATERIALS AND METHODS

Characteristic of test groups: The experimental protocol of this study was approved by the Research Ethics Committee, Faculty of Dentistry, Universitas Gadjah Mada (UGM) No. 001279/KKEP/FKG-UGM/EC/2018. The research was conducted over two months between January and March 2018 and involved 36 periodontitis rat models aged 2.5-3 months which were fed pellets and mineral water on a regular basis. Induction of periodontitis was effected by injecting 0.2 ml of *Aggregatibacter actinomycetemcomitans* bacteria intragingivally. Bacteria were injected into the labial mandibular incisors for seven consecutive days. The clinical signs of periodontitis in rats were observed as redness and enlargement of the gingiva and apically positioning of the gingival margin (gingival recession). The test population was divided into three groups: a treatment group (T: 20% snail mucin gel, n = 12), a positive-control group (P: hyaluronic acid gel/Gengigel®, n = 12) and a negative-control group (N: CMC-Na gel, n = 12).

Snail mucin processing: The snails (*Achatina fulica*) identified at the Animal Systematics Laboratory, Faculty of Biology, UGM were 5-10 cms in length and weighed 33 grams. Mucin gel was obtained from 20 subjects by means of a looped ligature being inserted in and pulled through their bodies. The gel obtained in this manner was collected in a glass beaker, its volume being measured before processing.

Snail mucin preparation: Snail mucin gel was made with 2% CMC-Na (2 grams of CMC-Na dissolved in 100ml of distilled water). A concentration of 20% was obtained by mixing 20ml of snail mucin with up to 100 ml of 2% CMC-Na and stirred for ten minutes before being transferred to a container and cooled to produce a gel subsequently sterilized with UV light.

The application of gel: 0.2 ml of the materials (20% snail mucin gel, hyaluronic acid/Gengigel®, CMC-Na gel) was topically applied to inflamed gingiva using a tuberculin syringe. The frequency of application was once daily for 14 days.

Observation and data analysis: The sample was stained with Haematoxylin-Eosin. The number of osteoblasts was observed on days 3, 5, 7 and 14 in five visual fields by two observers using a calibrated light microscope at 400x magnification. The normality of data was established by a Shapiro-Wilk test, while normality was evaluated by means of a Levene's test. All data was normally distributed and homogenous. A two-way ANOVA test was subsequently conducted to determine whether the test material, observation time or interaction between test material and observation time had any effect on the number of alveolar bone osteoblasts. The difference in the number of osteoblasts on days 3, 5, 7 and 14 was analyzed with a Post Hoc LSD test.

RESULTS

The results of histological observation in the treatment (T), positive control (P) and negative control (N) groups on days 3 (a), 5 (b), 7 (c) and 14 (d) can be observed in Figure 1. The number of osteoblasts in the study groups shown in Table 1 indicate that it increased between days 3 and 14. At all observation points, the highest number of osteoblasts was found in the treatment group. The lowest average number of osteoblasts was on day 3, while the highest was on day 14. The results of an ANOVA test indicated statistically significant differences between tested groups (p=0.000). The Post Hoc test confirmed that the highest number of osteoblasts was found in the treatment group followed by the positive control group.

DISCUSSION

Osteoblast as a crucial indicator of bone healing is expressed during the embryogenesis, remodelling and the bone healing process.[4] Morphologically, osteoblasts possess special characteristics in that they are cuboidal in shape and located at the interface of newly synthesized bone and strongly basophilic.[12] On the initiation of bone formation, osteoblasts synthesize basic substances and collagen which undergo polymerization which forms collagen fibers and tissue and, subsequently, osteoid. Osteoid constitutes a non-calcified matrix which does not yet contain minerals but which, shortly after deposition, is mineralized to become bone.[4] Therefore, the higher the number of osteoblasts present, the more rapid the bone healing process will be.

In this study, the results indicate that the number of osteoblasts increased during the observation period. This may, potentially, be due to the proliferation phase having started on day 3 when osteoblasts became active in the

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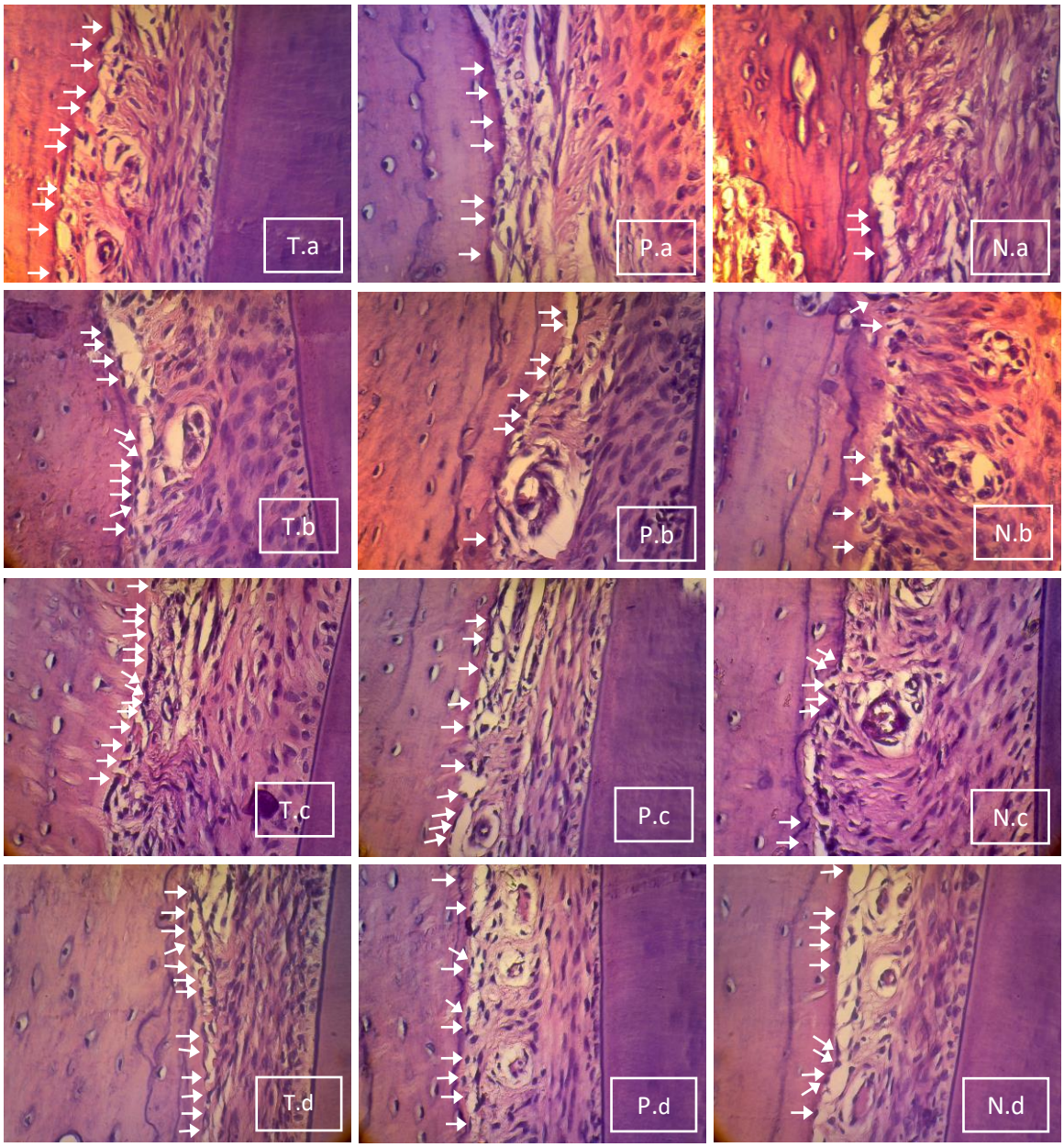

Figure 1. Osteoblasts of treatment group (T), positive control group (P), and negative control group (N) on day 3 (a), day 5 (b), day 7 (c), and day 14 (d). Osteoblasts are marked by white arrows. Picture taken at 400x magnification.

Table 1. The number of osteoblast (mean and standard deviation) in the treatment (T), positive control (P), and negative control (N) groups

Time (day)	The number of osteoblasts ($\bar{x}$ ± SD)			*p (intragroup)*	*p (intergroup)*		
	T	P	N		T-P	T-N	P-N
3	8.60±0.40	6.80±0.53	4.20±0.20	0.000*	0.000*	0.000*	0.000*
5	10.33±0.42	8.73±0.46	5.00±0.35	0.000*	0.000*	0.000*	0.000*
7	11.20±0.40	9.67±0.31	7.13± 0.58	0.000*	0.000*	0.000*	0.000*
14	12.13±0.31	11.20± 0.20	9.13±0.64	0.000*	0.000*	0.000*	0.000*

*statistically significant ($p<0.05$)

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bone formation process,[13] reaching its peak on day 14 as indicated by an increase in collagen and extracellular matrix deposition.[4]

The proliferative or granulation phase does not occur at a distinct time but, rather, continuously in the background. From day 5 to day 7, the fibroblasts started to lay down new collagen and glycosaminoglycans. These proteoglycans form the core of the wound helping to stabilize it. This phase lasts until the third week.[14] PDGF and TGF-β will help accelerate bone tissue formation by stimulating mitogenic activity and through the differentiation of osteoblasts and periodontal ligament fibroblasts.[15] Polypeptides of the TGF-β family are crucial to controlling cell activity and metabolism during ontogenic development in humans. These TGF-β family attributes are established during the bone healing process and considered to recapitulate embryonic intra-cartilaginous ossification.[16] In addition, PDGF and TGF-β will stimulate bone matrix deposition by osteoblasts. When detecting wounds, the body will automatically release bone morphogenetic proteins (BMPs). Disruption of this signaling will affect various skeletal and extra-skeletal anomalies.[15]

The highest number of osteoblasts was found both in the treatment and positive control groups. This contrast may have been due to the hyaluronic acid-containing gel contained in both these two groups. HA plays an important role in various biological cycles, including wound healing, chondrogenesis, immune response, cell migration[17,18] and osteogenesis.[9] Its oeteoinductive activity promotes growth factors such as bone morphogenetic proteins.[19] Interestingly, the number of osteoblasts in the treatment group was higher than positive-contol group. This might be due to other active substances contained in snail mucin gel such as glycosaminoglycans and glycoprotein. Glycosaminoglycans contain hyaluronic acid, heparan sulfate, heparin, chondroitin sulfate and hyaluronan sulfate, while glycoproteins contain achasin.[8,20]

Heparan sulfate (HS) is a membrane-bound proteoglycan featuring two main structures, namely; core protein and highly sulfated glycosaminoglycan side chains of D-glucuronic acid-N-acetyl-D-glucosamine repeats.[10,21] Heparin sulfate is a receptor for many substances such as coagulation enzymes, molecular adhesions, cytokines and proteases. This causes the heparane sulfate to execute a wide range of functions from supporting simple mechanical activities to supporting more complex processes such as cell proliferation and differentiation.[22] In the process of bone formation, heparane sulfate works by binding to bone morphogenetic protein (BMP). BMP ligands (e.g. BMP2, BMP4, BMP7) and their antagonist (Noggin) can bind to heparane sulfate because of their negatively charged side chains structure. These ligands and antagonists possess anti-and pro-osteogenic properties in bone. The process of binding HS with BMP and its antagonists can occur in one of two ways. The first is through restricted diffusion, during which BMP is transported from cell to cell by heparin sulfate. The second is heparin binding to the BMP antagonist to cause the inverse function of BMP. Increased BMP activity during bone healing will occur because these interactions can block the activity of inhibitors.[10]

Another snail mucin gel with the same structure and function as heparane sulfate is heparin. Heparin represents a glycosaminoglycan with a high sulfate content and extremely negatively charged molecules whose function is to influence BMP activity thereby affecting the bone formation process. Heparin can be found on the cell surface and in the extracellular matrix (ECM).[11]

Chondroitin sulfate is a component of sulfated glycosaminoglycans with two molecular structures, namely; N-acetylgalactosamine and glucuronic acid. With this molecular structure chondroitin sulfate can bind with various molecules such as growth factors, cytokines, chemokines, adhesion molecules and lipoproteins. The existence of these bonds supports the important role of chondroitin sulfate in cell growth, nerve development and tissue integration which supports anti-inflammatory activity and promotes the absorption of nutrients to cells.[23,24] Previous research has shown that chondroitin sulfate and sulfated hyaluronan can inhibit sclerostin and support bone regeneration in diabetic rats.[25]

Based on the results of a paper disc diffusion test, achasin could be seen to act as an antimicrobial against *Aggregatibacter actinomycetemcomitans* and *Streptococcus mutans* bacteria.[26] The benefits of the antimicrobial properties of achasin were observable on day 5 when pus was detected in the negative control group, but not in either the treatment or positive control group. CMC-Na present in the negative control group produced no antibacterial effect.[27] Previous research has also demonstrated that snail mucus promotes an increase in the density of collagen fibers during the gingival wound healing process due to the presence of glycosaminoglycans and glycoproteins.[28]

This study also confirmed that the topical application of 20% snail mucin gel induced osteoblast proliferation more rapidly than in other groups. The number of osteoblasts in the positive-control group on days 5, 7 and 14 was similar to that in the snail mucin gel on days 3, 5 and 7. Furthermore, the number of osteoblasts in the negative-control groups on day 14 was similar to that in snail mucus gel on day 3.

Within the limitations of this study, it is suggested that the topical application of 20% snail mucin (*Achatina fulica*) gel can increase the number of osteoblasts in a rat models with periodontitis. However, additional histological and clinical research is required to establish the role of this gel in bone regeneration in order that this gel can be used as an adjunctive therapy for periodontitis.

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