

The combination of carbonate hydroxyapatite and human β -defensin 3 to enhance collagen fibre density in periodontitis Sprague Dawley rats

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ABSTRACT

Background: Carbonate hydroxyapatite (CHA) is used as a scaffold to repair bone resorption. Alveolar bone resorption in periodontitis caused by an infection requires the presence of an antibacterial to support bone regeneration. Human β -defensin 3 (HBD3) is an antimicrobial peptide. The local application of the HBD3 antimicrobial is beneficial to inhibiting drug resistance and protecting tissue regeneration against invasive bacteria. **Purpose:** This study aims to investigate the effect of the administration of a combination of CHA with HBD3 on the collagen density of periodontitis rats (Sprague Dawley). **Methods:** This study was a true experimental study with a post-test control group design. Thirty-two Sprague Dawley animal models were randomly blind selected and placed under anaesthetic, then a 2-mm silk ligature was attached as a ligation to the mandibular incisors for 14 days in order to generate periodontitis. The study subjects were divided into two groups, the group with CHA and CHA-loaded HBD3 (CHA + HBD3) implantation. On days 7, 14, 21 and 28, four rats were taken randomly from each group for decapitation, followed by histological processing and examination with trichrome Mallory staining. The data was analysed using the Kruskal–Wallis test ($p < 0.05$). **Results:** An increase in collagen density during the healing process was found. There was a significant difference between CHA and CHA+HBD ($p = 0.004$ and $p = 0.008$; $p < 0.05$) in collagen density between the groups. **Conclusion:** The combination of CHA and HBD3 can enhance the collagen density in periodontitis Sprague Dawley rats, compared to CHA-only groups.

Keywords: carbonate hydroxyapatite; collagen; human β -defensin 3; periodontitis

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INTRODUCTION

Periodontitis is a periodontal tissue infection caused by a polymicrobial infection characterised by the destruction of the supporting tissue of the teeth.¹ One of the damaged periodontal tissues is the alveolar bone, which thereby requires surgical treatment and bone grafting to facilitate the formation of new bone tissue.² Bone grafts consist of three types, namely autografts, allografts, xenografts and synthetic grafts such as hydroxyapatite. This material has been widely approved as a biocompatible scaffold,

promoting osteoconduction of bone healing. It can be absorbed by the body.³

Hydroxyapatite includes a synthetic calcium phosphate material which is widely used as a bone graft. It has a similar chemical composition as the bone mineral matrix, producing superior biocompatibility, osteoconduction and osteointegration.⁴ Apatite is found in teeth and bone, and is 3-5% composed of carbonate ions, an arrangement of bone components called carbonate apatite (CO3Ap).⁵ Carbonate hydroxyapatite (CHA) is a biomimetic, biocompatible, biodegradable, osteoconductive bone

graft that promotes bone tissue without fibrotic tissue formation and stimulates osteogenesis.^{6,7} Based on previous studies, the biological activity of synthetic CHA is better than synthetic hydroxyapatite because the incorporation of carbonates in hydroxyapatite causes an increase in solubility and decreases crystallisation.⁶ CHA bone grafts can also be combined with various antibiotics, for example, metronidazole.⁸

The use of local and systemic antimicrobial therapy in extensive periodontal treatment is important as adjunctive therapy to prevent the growth and development of pathogenic bacteria.⁹ Human β -defensin3 (HBD3) is an antimicrobial cationic peptide with an immunomodulatory effect on innate and adaptive immune responses. The local application of an HBD3 antimicrobial is beneficial for protecting regenerated tissue against invasive bacteria that causes infection and for reducing the need for conventional antibiotics that cause drug resistance.¹⁰ Compared with other human defensins, HBD3 can reduce the formation of osteoclasts and reduce alveolar bone loss.¹¹ It functions as an osteogenic promoter through anti-inflammatory effects in the micro-inflammatory environment.¹² The combination of tissue techniques or synthetic bone grafts and antimicrobial therapy has strong potential for more successful tissue repair because infection is always a major risk factor during tissue regeneration.¹¹ Periodontitis therapy with bone grafts and the addition of local antimicrobial therapy increases bone regeneration and bone repair.^{12,13}

Collagen is the main structural component of the extracellular matrix as a positive indication of the healing development.¹⁴ Stages of bone formation start from the synthesis of type 1 collagen, collagen secretion, microfibril formation, fibrils and collagen fibers, maturation, the collagen matrix and the formation of hydroxyapatite crystals. All of these elements are under the influence of osteoblasts.¹⁵ The purpose of this study was to investigate the effect of the administration of a combination of CHA loaded with HBD3 in collagen density on Sprague Dawley rat with periodontitis condition.

MATERIALS AND METHODS

All the procedures of the *in vivo* experiment were approved by the Ethical Committee of the Faculty of Dentistry, Universitas Gadjah Mada, No.001511/KKEP UGM/EC/2018. The CHA was prepared by the Research Laboratory of the Faculty of Dentistry, Universitas Gadjah Mada, as described in the previous study.¹⁶ The membrane was prepared by a chemical mixture between carbonate hydroxyapatite and type B gelatin (MW 99 x 10³) from a bovine source (Nitta Gelatin, Osaka, Japan) and was carried out in sodium citrate solution at 37°C. The ratio of gelatin and CHA was of 7:3 (w.t %) respectively. The mixture was stirred continuously until a homogeneous solution was formed. Phosphate acid was dripped in the homogeneous solution and stored for 2 hours. After 2

hours, the homogeneous solution was dropped with 2 N HCl until the pH level reached 7.4. The gelatin solution containing CHA which has been chemically mixed was molded in 1gr/2025 mm² and was then stored in a freezer at 4°C overnight to dry and to form a membrane. When the membrane was dried, it was cross-linked with dehydrothermal at 140°C for 72 hours in a vacuum oven (Yamato ADP 200, Yamato Scientific Co., Ltd, Tokyo, Japan) and the gelatin CHA membrane was stored in an aluminium foil wrap at room temperature.¹⁶

HBD3 is an antimicrobial BD-3, a human recombinant SRP4524-20UG lyophilized solid (Sigma Aldrich, Saint Louis, MO, USA). The 10 μ l of HBD3 with a concentration of 12.5 μ g/l was loaded into 2 mg of membrane CHA.⁸

This research is a study using 32 healthy and active Sprague Dawley male rats, weighing between 300 and 400 grams, and aged 3 to 4 months. Experimental animals, Sprague Dawley rats, were adapted in a clean cage for 7 days. The rats were housed in each cage and maintained under a 12-hour light/dark cycle at a temperature of 23°C and a relative humidity of 50%, with access to standard rat chow pellets and water *ad libitum*. The Sprague Sawley rats were anaesthetised with ketamine (0.1 ml) and xylazine (0.1 ml) by an intramuscular injection in the thigh (dose 6–12 mg/kg). After the experimental animals entered the anaesthetic stage, a 2 mm silk ligature was attached as a ligation to the mandibular incisors for 14 days in order to generate periodontitis (Figure 1).¹⁷

The study subjects were divided into two groups, the group with CHA and the group with a CHA-loaded HBD3 (CHA + HBD3) implantation of the periodontitis in the mandibular incisor region. On days 7, 14, 21 and 28, four rats were taken randomly from each group for decapitation, followed by histological processing and an examination with trichrome Mallory staining and 40x magnification with an optical microscope Olympus BX 51 (Olympus, Tokyo, Japan). The criteria for evaluating the collagen fibers were set according to Tandelilin *et al.*¹⁸ as depicted in Table 1 and Figure 2. Collagen density was observed via the blind method. Each sample was prepared using a different code by a person who was not involved in the study.

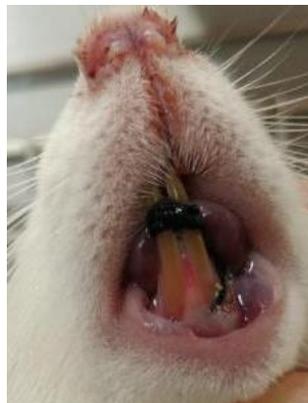


Figure 1. Day 14 ligation in Sprague Dawley rats.

The data was expressed as the mean \pm standard deviation. SPSS 20.0 software (IBM Corp., Armonk, NY, USA) was used for all the statistical analyses. After checking the data normality through the Shapiro–Wilk test ($p < 0.05$), when detects not normality data, the significant difference was calculated using the Kruskal–Wallis test ($p < 0.05$).

RESULTS

Means and standard deviations of the collagen density CHA and CHA-loaded HBD3 (CHA+HBD3) in each group was based on observation days (Figure 3). The Shapiro–Wilk test ($p < 0.05$) showed data normality. There were two data groups that were not normally distributed: the 14-day CHA

treatment group and 28-day CHA treatment group. On observation, days 7, 14, 21 and 28 showed that the density of collagen fibres in the implantation area of the CHA + HBD3 group was higher than the CHA-only group (Figure 4). The variance analysed using the Kruskal–Wallis test showed significant differences ($p < 0.05$) in collagen density between the treatment groups using CHA and the CHA-loaded HBD3 (Table 2).

DISCUSSION

The results on the 7th day showed that CHA and CHA loaded with HBD had formed collagen fibres of alveolar bone in the Sprague Dawley periodontitis rats.¹⁹ Collagen

Table 1. Scoring for collagen density used in the study

Score	
1	Collagen fibre density is less than 50% with less dense tissue structure, vascularisation, mononuclear cells, and many cells can be found.
2	Collagen fibre density is more than 50% with more dense tissue structure, less inflammatory reaction.
3	Avascular and acellular collagen fibrous density.

Table 2. Results of the Kruskal–Wallis test on the mean and standard deviation of collagen density in CHA and CHA + HBD3 groups

Implantation Time	CHA	CHA + HBD3
Day 7	22.00 \pm 1.414	26.50 \pm 1.291
Day 14	26.25 \pm 0.500	33.50 \pm 1.291
Day 21	27.25 \pm 0.957	34.25 \pm 1.258
Day 28	30.50 \pm 0.577	36.00 \pm 0.816
Asymp. Sig. (<i>p</i>)	0.004*	0.008*

* $p < 0.05$

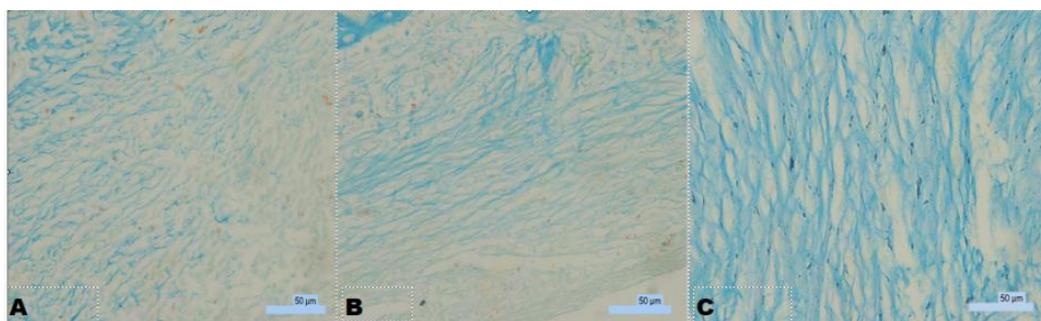


Figure 2. Collagen density score, Score 1 (A), Score 2 (B) and Score 3 (C).

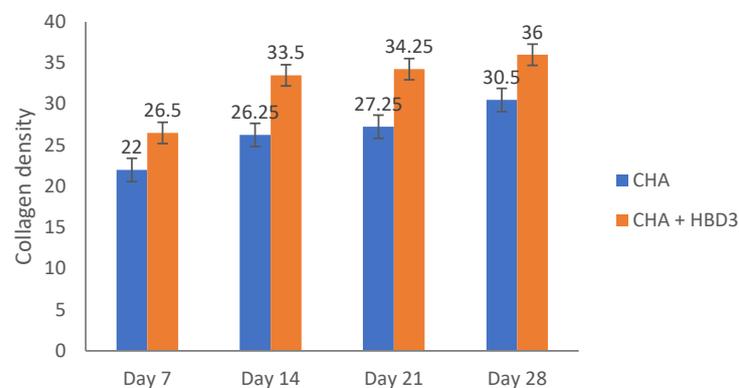


Figure 3. The increasing pattern of the collagen density fibre based on observation days between the CHA group and the CHA + HBD3 group.

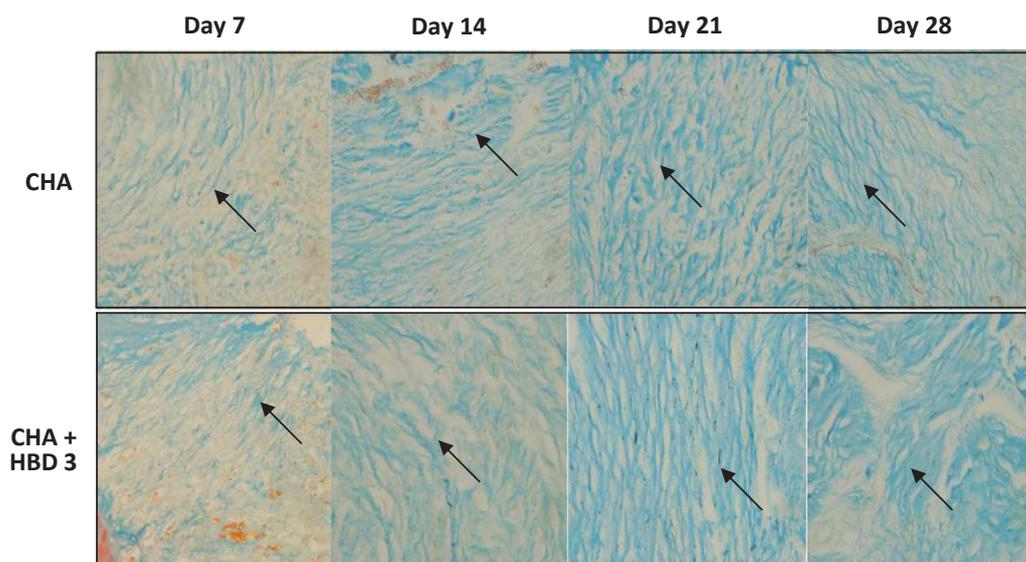


Figure 4. Histological findings of collagen density (black arrow) on observation days between the CHA and the CHA + HBD3 group by trichrome Mallory staining (magnification, 40x).

is a major protein that forms the extracellular matrix component, which is needed in the wound healing process and also needed in the formation of bone matrices.²⁰ A successful parameter in the wound healing process and bone formation process is the presence of collagen.¹⁹ On the 7th day, the transition phase of the blood vessel granulation tissue had been formed, with beginnings of fibroblast and fibrin tissue. Macrophages and cytokine regulation produce platelet-derived growth factors (PDGF), interferon gamma (IFN γ), fibroblast growth factors (FGF) and transforming growth factors (TGF- β) as growth factors that result in the reduction of fibroblasts to proliferate and migrate, and to produce extracellular fibres such as collagen, elastin fibres, and reticular fibres that needed for cutting processes. CHA and HBD3 membranes have the same properties that affect the proliferation of fibroblasts.^{21,22} Fibroblast cells play an important role in collagen synthesis, depositing and renovating connective tissue and regenerating bone tissue. Collagen synthesis increases in the damaged area²¹ and results in the complication on day 7 relating to both CHA and CHA + HBD3.

On days 14 to 28 after the implantation, there was an increase in collagen fibre and collagen synthesis. The results of this study were not consistent with the previous theory stating that the formation of collagen fibres begins on day 3 and peaks on day 7, then decreases between days 14 and 21.²⁰ However, the results of this study are in line with research by Ardhiyanto and Siswomihardjo²³ that shows that after the implantation of hydroxyapatite on the 14th day, the amount of collagen continues to increase until the 28th day. This condition is caused by the CHA providing the right microenvironment to repair bone resorption of tissue²⁴ and to eliminate the inflammation.²⁵ Thus, collagen becomes more dense and alveolar bone regeneration occurs. On days 21 to 28, a bone remodelling process occurs that

regulates the balance of bone formation and resorption. The purpose of the remodelling phase that occurs from day 21 to one year after the injury is to complete tissue repair and to restore tissue integrity.^{21,26}

The results of the study on all days showed that the periodontal regeneration of the CHA + HBD3 group was higher than the CHA group, characterised by thicker and denser collagen in the CHA + HBD3 group. This might happen because of the interaction between the CHA, which has high osteoconductive properties, so it could stimulate new bone growth⁵ and the HBD3 that has antimicrobial properties, promoting regeneration in the inflammatory environment. Moreover, CHA + HBD3 also increases cellular activity, provides cells that stimulate differentiation of the extracellular matrix, which synthesises the development of new tissue to increase tissue osteogenic differentiation and periodontal regeneration.^{21,26,27} The addition of HBD3 to the CHA membrane is necessary because pathogenic microbes that adhere to the tooth surface and contaminate periodontal lesions are confounding factors in the regeneration of periodontal tissue. Infection control could be carried out to optimise the regeneration process.²⁸

The antibacterial scaffold (CHA + HBD3) makes the extracellular matrix situation in the damaged tissue change quickly in order to accelerate the healing process and the regeneration of alveolar bone. Limitation in the research lies in the limited HBD3 material and the long delivery process. In conclusion, a combination CHA and HBD3 could enhance the collagen density in the periodontitis animal model compared to the CHA group only. It is suggested for the future research to investigate the variables in a longer time frame, approximately three months, and to investigate more of the osteoblast and osteoclast variables in order to see alveolar bone regeneration.

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