ORIGINAL ARTICLE

Doxycycline Incorporated in Gelatin - Carbonate Apatite Bone Graft Material: In Vitro Evaluation of Osteoblastic Alkaline Phosphatase and Porphyromonas gingivalis Colonies

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ABSTRACT

Introduction: Periodontitis treatment should promote the bone regeneration under minimal infection risks during the healing. The incorporation of local antibiotic doxycycline into bone graft material could prevent the bacterial attachment which could lead to failure in the regeneration process, but could decline the alkaline phosphatase (ALP) level. Incorporation of doxycycline into carbonate hydroxyapatite–gelatin bone graft should not affect the effectiveness of the antibacterial as well as regenerative properties. The antibacterial effect on Porphyromonas gingivalis and the alkaline phosphatase (ALP) was be evaluated. This study aimed to evaluate the profile of P. gingivalis colony growth and ALP from osteoblast cultures after contact with a doxycycline–carbonate hydroxyapatite–gelatin bone graft. Methods: Dry specimens of 10 mg alloplastic carbonate apatite were embedded with (0.05 %, 0.1 %, and 0.15 %) doxycycline solution for 24 h. In 96-well plates, the specimens were embedded into colonies of P. gingivalis, and then they were moved into a new well of colonies every 24 h. The concentration of colonies was evaluated using a microplate reader. The other specimens were immersed in osteoblastic MCT3T3 culture cells, and then the osteoblast ALP was evaluated. Results: The concentration of P. gingivalis colonies was inversely proportional to the doxycycline concentration. The ALP concentrations showed no statistical difference between groups. Conclusion: These findings demonstrated that doxycycline was incorporated into the gelatin–carbonate apatite material and showed an antibacterial effect up to 72 h but does not alter the effect of ALP in the osteoblast.

Keywords: Carbonate apatite, Doxycycline, Alkaline phosphatase, Porphyromonas gingivalis

INTRODUCTION

Alloplastic bone graft materials have an inherent risk of bacterial infection (1). Thus, an antibiotic is needed to decrease the risk of bacterial infection (2). Moreover, reducing oral bacterial biofilm requires a higher concentration of antibiotic compared with that of planktonic biofilms. However, high doses of systemically administered antibiotics have several risks, like the development of antibiotic-resistant strains and lower bioavailability because of the systemic route of metabolism (3). Thus, local routes of delivery exhibit fewer side effects and directly influence the site of injury; however, the actual drug availability at the site needs to be evaluated (4).

The attachment of bacteria to the bone graft surface leads to bacterial colonization and induces immune responses (5). Bacteria can induce the activation of host proteolytic enzymes, such as matrix metalloproteinase (MMP), which are detrimental to the success of the bone graft (6). Doxycycline has been confirmed not only as an antibiotic agent but also as an anti-inflammatory and immunomodulatory agent (7).

A combination of antibiotic agents and alloplastic bone graft materials is used in orthopedics to prevent bacterial adhesion and post-surgical infection (8). Thus, it might also be the right combination for periodontal therapy, as it can fulfill the treatment goals, which focus on the eradication of bacteria and enhancement of new bone formation (6). Carbonate apatite–gelatin has been proven as an alloplastic bone graft material, and a delivery agent or drug delivery system (DDS). Controlled release is expected to provide an adequate local therapeutic effect at the sites of bone graft (9).

Incorporating apatite carbonate with antibiotics requires consideration in determining the release of antibiotic ingredients or drugs. Also, antibacterial trials of a DDS with antibiotics need to be done to determine
their therapeutic properties (10). The incorporation of antibiotics into bone graft materials needs to consider loading the active ingredient in the carrier, in this case, a bone graft. The release of antibiotics from the doxycycline-incorporated bone graft materials is expected to occur gradually over a long period (11). The release of doxycycline has effect on the ALP of the osteoblast, which is beneficial for periodontal treatment (12). In the other hand, the addition of antibiotic into bone graft material could also decreasing the ALP (13). The DDS of doxycycline–carbonate hydroxyapatite–gelatin bone graft should not affect the effectiveness of both materials (14). This study aimed to evaluate the \textit{P. gingivalis} colony profile and ALP from osteoblast culture after contact with a doxycycline–carbonate hydroxyapatite–gelatin bone graft.

**MATERIALS AND METHODS**

**Materials**

Graft material (GAMA-CHA, Kimia Farma, Indonesia), doxycycline hyclate, \textit{P. gingivalis} ATCC33277 strain, PBS (Phosphate Buffered Saline) (1X, pH 7.4), osteoblastic MC3T3 cell culture, ELISA reader (iMark, BioRad, USA), analytical balances (Mettler Toledo, Swiss), magnetic stirrer, centrifuge (Centrifuge 5415D, Eppendorf, USA), micropipettes, pH meter, incubator (37 °C), and microplate.

**Materials and Methods**

This research was granted ethical approval by the Research Ethics Committee, Faculty of Dentistry, Universitas Gadjah Mada. (001276/KKEP-UGM/EC/2017). All tests were performed in triplicate.

**Doxycycline solution preparation**

The doxycycline solution was prepared by dissolving each of 5 mg, 10 mg, and 15 mg of doxycycline hyclate (Doxycyline Hyclate, Sigma Aldrich) in 100 ml of PBS solution using a magnetic stirrer. Then, the pH of the solution was measured and adjusted to achieve a pH value of 7 to 7.4.

**Doxycycline–carbonate hydroxyapatite–gelatin bone graft preparation**

Specimens of the bone graft material (GAMA-CHA, Kimia Farma, Indonesia) were prepared by cutting the bone graft material into sections weighing 10 mg and then storing each section in a microcentrifuge tube. Each specimen was embedded in 1 mL (0.05%, 0.1%, and 0.15%) doxycycline solution for 24 hours. The specimen was then dried and was sterilized using ethylene oxide gas.

**Antibacterial test**

The antibacterial test was performed using the \textit{P. gingivalis} colony (ATCC 33277) in brain heart infusion (BHI) medium. The test was initiated by adding 10 µL of \textit{P. gingivalis} and 200 µL of BHI solution into a 96-well plate. A 10 mg specimen was added into the well, and the other wells were left with no specimens as controls. The microplate was then incubated in an anaerobic jar at 37 °C for 24 h. After 24 h, the specimens were moved to another well contain new colony, then the previous colony concentration was measured by determining the absorbance of \textit{P. gingivalis} colonies using a microplate reader (iMark™, Bio-Rad Laboratories, USA) set at a wavelength of 595 nm. The specimens were incubated into the new well for another 24 h, then the same procedures were done for one more cycle, for a total of 96 h. The optical density for each well was compared with the control.

**Alkaline Phosphatase**

Osteoblastic cells (MC3T3) were cultured in a 24-well plate using Eagle’s minimum essential medium to alpha modification (α-MEM). The cells were cultured in 1 mL medium, and then the 0.4 µM Corning® Transwell® polycarbonate membranes cell culture was inserted along with the specimens, in order to separate the specimens and the culture. Then, 500 µL medium was put on top of the membrane. The specimens were co-cultured with the cells and incubated for seven days. The ALP assay was performed colorimetrically according to the protocol of the ALP colorimetric assay kit manual (BioVision Inc., CA).

**RESULTS**

The results of antibacterial testing are shown in Fig 1. The results indicated that all groups could release doxycycline for up to 96 h. The lowest bacterial concentration was specimens incubated for 24 h; then bacterial concentration was increased proportionally to incubation times. All doxycycline-containing specimens showed lower bacterial concentrations compared with the control up to 96 h (p < 0.05). There was no statistically significant difference between the 0.05 % and 0.1 % groups. However, the highest concentration of doxycycline incorporated into the gelatin–carbonate apatite showed the lowest concentration of bacteria on all days (p < 0.05). The concentration of \textit{P. gingivalis} increased in all groups.

![Image](image-url)
The result of ALP activity in all groups showed no statistically significant difference \((p > 0.05)\). ALP was measured by total ALP and the percentage from the control group. Fig 2 shows the total ALP count from all groups. The highest total amount of ALP showed in 0.1% group, followed by 0.15% and 0.05%, respectively. Fig 3 illustrates the ALP percentage concentration with the control group as the reference (100%).

The concentration of 0.15% has the highest doxycycline content. At 96 h, only 0.15% of doxycycline had a lower bacterial concentration compared with the control. The antibacterial effects of antibiotics combined with bone graft materials will be directly proportional to the concentration of antibiotics in the bone graft (3). The other groups showed a higher concentration of bacteria but did not explain why the specimens would increase the growth of bacteria. In contrast, these findings only correlated with the inability of bacterial inhibition.

Alkaline phosphatase (ALP) activity showed no change in all groups. Bone-specific alkaline phosphatase is a biomarker of osteoblast activity and bone formation (21). A previous study showed that the addition of doxycycline, minocycline, and tetracycline to osteoblast cell culture has a dose-dependent effect. The present study showed that the addition of doxycycline into bone graft material did not affect the ALP activity in the cell, even in the highest concentration. Thus, the addition of doxycycline into the bone graft materials did not alter the activity of osteoblasts, observed with ALP (12).

The antibacterial and ALP amount evaluation showed that specimens doxycycline–carbonate hydroxyapatite–gelatin bone graft could be a candidate for antibiotic loaded alloplastic bone graft materials. Doxycycline’s roles in osteogenesis were also shown on the surface of bone implants (22). Doxycycline in implant surface could improve the healing of intrabony defects, compared to bone graft alone (24). Doxycycline could also downregulate osteoclastogenesis, which would be beneficial in the regeneration of bone in periodontal defects. Some studies showed that doxycycline reduces the release of collagenase enzymes, such as MMPs (25).

CONCLUSION

This study demonstrated that the incorporation of doxycycline into gelatin carbonate apatite alloplastic bone graft material has antibacterial activity. It also showed that the ALP concentration in osteoblast cell culture is not altered by doxycycline. This study is the pilot research for evaluation of antibiotic loaded into alloplastic bone graft material for periodontitis treatment. The study limited only evaluated the \(P.\ gingivalis\) colony and ALP amount in vitro.
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REFERENCES