ORIGINAL ARTICLE

# In Vitro Evaluation of Doxycycline Incorporated in Carbonate Apatite – Gelatin Bone Graft Material

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**ABSTRACT**

**Introduction:** Bacterial adhesion is a factor responsible for alloplastic bone graft failure. The non-vascularized structure of the alloplastic carbonate apatite and exposure to the oral cavity could lead to bacterial plaque attachment, resulting in failure of the regeneration process. Incorporation of antibiotic into the bone graft material is expected to increase the effectiveness of eradication of bacteria. Objective: to evaluate the incorporation of doxycycline in carbonate apatite – gelatin bone graft material. **Methods:** Specimens of 10 mg alloplastic carbonate apatite bone grafts were immersed in 0.05%, 0.1%, and 0.15% doxycycline solutions (1 ml) for 24 hours. Doxycycline loading percentage, its release percentage profile, antibacterial tests on *Porphyromonas gingivalis*, and fibroblast viability tests using MTT assay were performed. **Results:** The highest loading percentage was obtained from lowest concentration (0.05%) of doxycycline solution. The release profiles of doxycycline among the three groups were not significantly different. The cytotoxic effect of doxycycline was not observed up to 0.15%. Antibacterial effect on *P. gingivalis* was found to be in a concentration-dependent manner. **Conclusion:** These findings demonstrated that bone graft material based on 0.15% doxycycline-incorporated apatite carbonate is not toxic and has a bacterial inhibitory effect.

**Keywords:** Alloplastic, Bone graft, Doxycycline, Local delivery system

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## INTRODUCTION

Alloplastic bone graft materials inherit the risk of bacterial infection[1]. Thus, an antibiotic is needed to decrease the risk of bacterial infection [2]. Moreover, reducing oral bacterial biofilm, needs higher concentration of antibiotic compared to the planktonic ones. However, high doses of systemically administered antibiotics have several risks like development of antibiotic-resistant strains and lesser bioavailability due to the systemic route of metabolism [3]. Thus, local routes of delivery exhibit lesser side effects and directly influence the site of injury; however, the active drug availability at the site needs to be evaluated [4].

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 for the success of 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 consequent post-surgical infection [8]. Thus, it might also be a good combination for periodontal therapy, as it can fulfill the treatment goals, which focus on eradication of bacteria and enhancement of new bone formation [6]. Carbonate apatite–gelatin has been proven as alloplastic bone graft material, as well as 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. In addition, antibacterial trials of a DDS with antibiotics also need to be done

for determining their therapeutic properties [10]. Incorporation of antibiotics into the bone graft materials needs to consider the process of 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 of time [11]. Additionally, the effectiveness of an incorporated antibiotic should not be lost, and the material should be non-toxic to the environment surrounding the tissue defect [8]. This study aimed to understand the antibacterial and biocompatibility properties of doxycycline incorporated into carbonate apatite-gelatin bone graft material.

## MATERIALS AND METHODS

### Materials

Graft material (GAMA-CHA, Indonesia), doxycycline hyclate, Porphyromonas gingivalis ATCC33277 strain, phosphate buffered saline (PBS) solution, UV–Vis spectrophotometer (UV1800, Shimadzu, Japan), 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.

### Methods

This research was granted an ethical approval by the Research Ethic Committee, Faculty of Dentistry, University of Gadjah Mada. (001276/KKEP-UGM/EC/2017).

### Doxycycline solution preparation

The doxycycline solution was prepared by dissolving each of 5, 10, and 15 mg of doxycycline powder in 100 ml of PBS solution using a magnetic stirrer. The pH of the solution was measured and adjusted to achieve a value of 7–7.4.

### Doxycycline loading percentage

The method of incorporation and measuring the amount of doxycycline was performed in accordance with the previous studies [6, 8, 10]. The doxycycline charge calculations were performed by immersing bone graft specimens in 10 ml doxycycline solution (0.05%, 0.1%, and 0.15%) for 24 hours. Measurement of loading percentage was determined by calculating the absorbance values using UV–Vis spectrophotometer at 273 nm, before and after immersion:

$$\text{Doxycycline Loading percentage} = \frac{(A - B)}{A} \times 100\%$$

A: absorbance of doxycycline solution before immersion
B: absorbance of doxycycline solution after immersion

### Doxycycline release percentage

Specimens of doxycycline-incorporated bone graft materials were then dried in 37°C incubators with filter paper lids. Each dried specimen was immersed in PBS solution in a microcentrifuge tube. After 30 min, the solution and specimen were centrifuged at 2,000 rpm for 5 min. The solution was then moved into the cuvette using a micropipette, while the bone graft specimen was re-immersed in a fresh PBS solution [11]. The absorbance value of the solution was measured using a UV–Vis spectrophotometer with a wavelength of 273 nm. The procedure was repeated at intervals of 1, 2, 3, 24, and 48 hours.

### Antibacterial effect

The antibacterial effect was assessed using a diffusion technique. *Porphyromonas gingivalis* ATCC 33277 was adjusted at 108 CFU/ml in accordance with the Mc Farland's standard. The bacterial cultures were then dissolved in a 10 ml Brain Heart Infusion liquid broth. The bacterial suspension was swabbed on the surface of the Mueller Hinton Agar (MHA) medium in a petri dish. Holes were made in accordance with the diameter of bone graft material, and doxycycline-incorporated bone graft material was put in each hole. The MHA plate was incubated in an anaerobic incubator at 37°C for 24 hours, and the inhibitory zone was measured.

### Fibroblast viability

The fibroblast cells used were primary fibroblast cell cultures. Primary periodontal ligament fibroblast cells were isolated from root surfaces of extracted teeth and cultured in the Dubelco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum, 2% penicillin-streptomycin, and 2% fungizone, in culture flasks. The highest concentration of doxycycline (0.15% was used in this test doxycycline diluted up to eighth 2-fold dilutions. The test material was inserted into the well, and the fibroblasts were incubated at 37°C. MTT assay was performed by calculating optical density value measured with Elisa reader.The percentage of living cells was calculated by:

$$\%\ \text{living cells} = \frac{\text{Optical density of test well}}{\text{Optical density of positive control well}} \times 100\%$$

### Statistical analyses

Doxycycline load percentage data, release of doxycycline, bacterial zone of inhibition, and percentage of living cells were tested for normality and homogeneityStatistical tests were applied using one-way ANOVA followed by post hoc test, non-parametric test was applied on the abnormally distributed but homogenous data.

## RESULTS

### 1. Doxycycline Loading Percentage

The highest percentage of loading (61.14%) was found in graft embedded in 0.05% doxycycline solution (Table I),followed by specimen embedded in 0.10% (51.13%) and 0.15% doxycycline solution (40.63%). One-way ANOVA test results showed p = 0.002 and indicated that the mean difference between the different concentration groups was statistically significant with a significance level of 0.05. Post hoc test results showed that all the groups had $p < 0.05$ indicating that significant differences occurred among all the groups.

**Table I : Average and standard deviation of doxycycline loading percentage on carbonate apatite–gelatin bone graft**

| Groups | N | Loading (%) |
|---|---|---|
| A | 5 | 61.14 ± 3.07 |
| B | 5 | 51.13 ± 5.21 |
| C | 5 | 40.63 ± 9.98 |

A : carbonate apatite–gelatin bone graft and 0.05% doxycycline solution
B : carbonate apatite–gelatin bone graft and 0.10% doxycycline solution
C : carbonate apatite–gelatin bone graft and 0.15% doxycycline solution

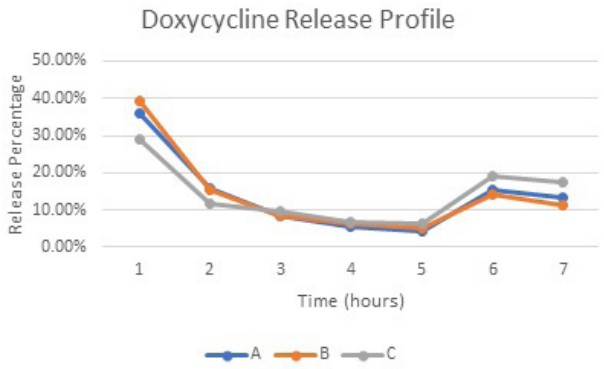


**Figure 1 :** Doxycycline release profile.

### 2. Doxycycline Release Percentage

The maximum doxycycline release among all the three concentration groups was found in the first 0.5 hours and continued up to 48 hours (Figure 1). The release percentages in all the groups decreased gradually up to 4 hours. Starting off at 2 hours, specimen embedded in 0.15% doxycycline solution had a higher release percentage than the other two groups and showed highest values among all the groups until 48 hours. The maximum rates of changes of absorbance were found in the 24-hour interval between 24 hours and 48 hours. Hence, the result showed prolonged antibiotic release from the bone graft materials.

The doxycycline release profile is shown in Figure 1 presents the percentage of doxycycline release in PBS after incubation at several points of time. The result of Kruskal–Wallis test showed p = 0.769 ($p > 0.05$), indicating no differences in means among all the groups. These results meant that the concentration of the doxycycline solution during immersion process has no effect on the doxycycline release percentage.

### 3. Antibacterial Effect

The largest inhibition zone of 14.87 mm was observed in specimen embedded in 0.15% doxycycline solution (Table II). The results of one-way ANOVA statistical test showed p = 0.000 ($p < 0.05$), indicating that there were statistically significant differences among all the groups. The result of post hoc test, the Least Significant Difference (LSD) method, showed $p < 0.05$, indicating that the groups had significant mean differences among them, and that specimen embedded in 0.15% doxycycline solution had the greatest inhibitory effect, followed by specimen embedded in 0.10% and 0.05% doxycycline solution, respectively.

**Table II : Average and standard deviation of *P. gingivalis* inhibition zone**

| Groups | n | *P. gingivalis* Inhibition zone (mm) |
|---|---|---|
| A | 3 | 9.80 ± 0.20 |
| B | 3 | 12.03 ± 0.15 |
| C | 3 | 14.87 ± 0.31 |

A : carbonate apatite–gelatin bone graft and 0.05% doxycycline solution
B : carbonate apatite–gelatin bone graft and 0.10% doxycycline solution
C : carbonate apatite–gelatin bone graft and 0.15% doxycycline solution

### 4. Fibroblast Viability

The viable percentage of fibroblasts from the treated groups was obtained by comparing the number of viable cells and positive controls (Table III). The smallest viable-cells percentage of 83.05% was found in the largest dose concentration group. From the fifth to the ninth 2-fold serial dilution, the mean viable-cells percentage exceeded 100%, indicating that the number of viable cells in the well was higher than that in the positive control well. The results of one-way ANOVA test showed p = 0.000 ($p < 0.05$), indicating that there were statistical differences in the averages of viable-cells percentage. The results of the LSD post hoc test showed that there were significant differences with the reduction in dose concentration (Table IV).

**Table III : Average and standard deviation of viable-cells percentage of fibroblasts**

| Group | A | B | C | D | E | F | G | H | I |
|---|---|---|---|---|---|---|---|---|---|
| N | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| Cell viability | 83.05± 0.48 | 90.35± 10.62 | 95.99± 10.77 | 97.65± 7.42 | 100.76± 1.64 | 102.56± 10.31 | 106.92± 10.31 | 121.18± 6.05 | 126.90± 7.99 |

a: 0.15% doxycycline solution;
b: first 2-fold diluted solution x 1/2;
c: second 2-fold diluted solution x 1/4;
d: third 2-fold diluted solution x 1/8
e: fourth 2-fold diluted solution x 1/16
f: fifth 2-fold diluted solution x 1/32
g: sixth 2-fold diluted solution x 1/64
h: seventh 2-fold diluted solution x 1/128
i: eighth 2-fold diluted solution x 1/256

**Table IV : Least Significant Difference (LSD) post hoc test**

| | A | b | c | d | E | F | g | h | I |
|---|---|---|---|---|---|---|---|---|---|
| **A** | | 0.345 | 0.103 | 0.068 | 0.030* | 0.018* | 0.005* | 0.000* | 0.000* |
| **B** | | | 0.462 | 0.344 | 0.183 | 0.122 | 0.041* | 0.001* | 0.000* |
| **C** | | | | 0.828 | 0.535 | 0.395 | 0.164 | 0.004* | 0.001* |
| **D** | | | | | 0.685 | 0.523 | 0.234 | 0.006* | 0.001* |
| **E** | | | | | | 0.814 | 0.424 | 0.014* | 0.003* |
| **F** | | | | | | | 0.569 | 0.023* | 0.005* |
| **G** | | | | | | | | 0.074 | 0.0168 |
| **H** | | | | | | | | | 0.457 |
| **I** | | | | | | | | | |

*) $p < 0.05$
a: 0.15% doxycycline solution;
b: first 2-fold diluted solution x 1/2;
c: second 2-fold diluted solution x 1/4;
d: third 2-fold diluted solution x 1/8
e: fourth 2-fold diluted solution x 1/16
f: fifth 2-fold diluted solution x 1/32
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## DISCUSSION

The inverse ratio between the concentration of the solution and the percentage of charge was in accordance with the previous study [8]. It is also considered that porosity and surface area have an influence on drug absorption by this kind of immersion technique. The degree of saturation of the drug at each time correlated with a lower dose of active ingredient uptake from the higher concentration of the solution. The higher doxycycline immersion solution correlates with lower loading percentage. In this research, we did not measure the exact amount of doxycycline in the bone graft material. It was assumed that even if the loading percentage of 0.15% doxycycline is lower than 0.05%, the amount of doxycycline inside the bone graft material immersed in 0.05% could not be assumed higher than 0.15% [7]. The bonds that occur between antibiotics and the carbonate apatite–gelatin bone graft are hypothetically physical and chemical bonds [8, 12-15].

The release profile presenting the amount of antibiotic drug released from bone graft materials is affected by the carrier, medicinal properties,

and antibiotic concentrations [3]. Therefore, the degradation profile of the bone graft material affects the percentage of drug release [10]. Gelatin is a biocompatible and biodegradable material, with its degree of biodegradability adjustable through different cross-linking processes [16]. The apatite carbonate has a potential to act as a vehicle or carrier in DDS [17, 18].

The release of the doxycycline antibiotic was still observed for up to 48 hours, indicating a gradual release of doxycycline [9, 19]. A high discharge in the initial 0.5 hour indicates a high concentration of doxycycline in the environment in vitro. A high release at the beginning, so called a burst release, is useful for eradicating the postoperative local bacterial infection [20]. Moreover, the doxycycline concentration in case of local application gets reduced gradually. Due to the same delivery sistem, release profile of all the groups is not statistically different. The release profile are according to the degradation of the material used for the delivery system [10].

The antibacterial test used in this study was the diffusion method. The diffusion method of the wells is useful for determining whether an ingredient can inhibit the growth of certain bacteria and is in accordance with the principle of early screening of antibacterial effects [21]. It has been demonstrated by the bacteriological viability test that a local doxycycline application in implant therapy can eradicate *P. gingivalis* [22]. The antibacterial effect of bone graft material with antibiotics has also been studied by Dashti et al. to establish that the antibiotics eradicate bacteria and inhibit bacterial growth [8]. Both materials help in the process of DDS, thus eradicating the wash-out in a short time [23].

The antibacterial property of a local antibiotic material should be accompanied by non-toxic properties. The lowest viable cell percentage (83.05%) was found in the highest doxycycline concentration of 0.15%. According to ISO 10993-5,when the viable cell count is reduced by 30% more than the control, in other words the growing cells percentage is below 70%, a substance is considered as toxic [24]. In accordance with this statement, the doxycycline concentrations used in this study did not have toxic properties even at the highest concentrations.

The proliferative effectiveness of doxycycline in small doses on other cells of the periodontal tissue as the cells targets was evaluated. At dilution to small concentrations, the growth of periodontal ligament cells was higher compared with the positive controls. This result suggests that doxycycline has a proliferative ability against periodontal ligament tissues. Potential proliferative properties of doxycycline on periodontal ligament cells and keratinocytes have been reported [22]. This study indicated that a doxycycline concentration between 20–100 µg/ml has a proliferative ability toward periodontal ligament cells in vitro. A 1–200 µg/ml concentration also does not change the morphology of cells and cell viability. The concentrations of 20 µg/ml can specifically increase the activity of DNA synthesis.

## CONCLUSION

Based on these four tests, the best characteristics of doxycycline-incorporated apatite carbonate bone material were obtained at the concentration of 0.15%. This concentration also showed good physical characteristics, i.e., optimal load and controlled release. In addition, biological characteristics, i.e., effective antibacterial activity and non-toxic effect on cultured fibroblasts from the periodontal ligament have also been shown.

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