EFFECTIVENESS OF CHITOSAN NANOPARTICLE CONTAINING 0.7% TETRACYCLINES ON CLINICAL PARAMETERS AND FIBROBLAST GROWTH: FACTOR-2 IN RAT MODELS

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ABSTRACT

Objective: Tetracyclines in periodontitis improved probing depth, increased attachment level, and reduced bacterial colonies. Chitosan nanoparticles have slower drug release, better drug stability, and lower toxicity. This study is to evaluate the effectiveness of subgingival application of chitosan nanoparticles containing 0.7% tetracyclines in the periodontitis model on clinical parameters and fibroblast growth factor-2 (FGF-2) expression.

Methods: Laboratory experimental research on 29 Wistar rats induced by p. gingivalis with pretest posttest control group design, divided into 4 treatment groups, 2 placebo groups, and 1 negative control group. Clinical parameters of bleeding on probing (BOP) and PPocket Depth (PD) and FGF-2 expression were evaluated on day 3 and 8.

Results: Significant decrease in BOP was only found in the daily application group of chitosan nanoparticles containing 0.7% tetracyclines on day 8 (p=0.008) and significant decrease in mean PD was found in the application group of chitosan nanoparticles containing 0.7% tetracyclines every day (p=0.034) or only once (p=0.046). FGF-2 expression increased on day 3 of each group and decreased on day 8 of each group. Significant difference in the FGF-2 expression was found in all groups on day 3 (p=0.034) but it was only between the daily application group and the once only application group (p=0.012).

Conclusion: There was a decrease in the percentage of BOP and PD in all groups as well as a significant increase in FGF-2 expression on day 3 and 8 after the application of chitosan nanoparticles containing 0.7% tetracyclines.

Keywords: Chitosan nanoparticle containing, Fibroblast growth.

INTRODUCTION

The term periodontal disease covers various degenerative and inflammatory conditions of gingiva, periodontal ligament, cementum and alveolar bone [1]. This disease is considered as one of the two main threats to oral health [2]. Riskesdas in 2018 shows the percentage of periodontitis in Indonesia generally reaches 74.1%. In terms of age, the greatest prevalence of periodontitis is in the 25-44 age group, reaching 77.0% [3].

Tetracyclines have been widely used in treating refractory periodontal disease, including localized aggressive periodontitis. Tetracyclines are able to remain in periodontal tissue and inhibit A. actinomycescomitans from growing. In addition, tetracyclines exhibit anti-collagenase effects which can inhibit tissue destruction and help bone regeneration [4].

Systemic administration of tetracyclines after scaling-root planing can eliminate tissue bacteria and has been shown able to stop bone loss and suppress microbial levels [4]. However, systemic administration can only achieve relatively low concentrations in the pocket even when given in high doses to patients. The development of controlled subgingival drug delivery systems has enabled the maintenance of effective intrapocket levels of antibacterial agents for long periods of time and has been shown to alter the subgingival flora and influence the healing of the attachment apparatus [5].

Sharma et al. in their research using tetracyclines as a local drug system to treat chronic periodontitis showed improvement in probing depth, attachment level, as well as a decrease in bacterial colonies significantly compared to the control group [6]. This indicates the placement of antibiotics or antiseptics directly on the root surface can reduce or eliminate pathogenic organisms that cannot be removed mechanically [5].

Chitosan hydrogel as a natural polymer has attracted much attention in recent years. Chitosan is a kind of natural polysaccharide cellulose that is non-toxic, biocompatible and biodegradable. Chitosan was chosen as a transport for tetracyclines because it has other useful bioactivities such as anti-inflammatory properties [7]. Popa L, Ghica M, Elena C in research on the use of chitosan as a gel material stated that chitosan is considered as an adequate local drug release system in the periodontal pocket. This material can remain in periodontal pocket and the release of antimicrobial agents in the crevicular fluid can be controlled [8].

Research by Susanto C et al. shows that chitosan can be used as a topical drug conductor. Chitosan material can release tetracyclines in the chitosan gel. The average diameter of the chitosan-based 0.7% tetracyclines gel inhibition zone is more than 27 mm which indicates very strong antibacterial activity [9] and in research by Andrew et al., non-toxic properties to fibroblast cells have been demonstrated [10]. Astuti WD in research on a rat model of periodontitis also succeeded in showing an increase in collagen density after scaling-root planing accompanied by the application of 0.7% tetracyclines based chitosan hydrogel [11].

Nanoparticulate system has several advantages compared to microspheres, microparticles and emulsion-based delivery systems, including high dispersibility in aqueous media, controlled release rate and increased stability [12]. El-Aly et al. in their research developed a form of chitosan nanoparticles that has higher antibacterial activity than chitosan. Chitosan nanoparticles provide several advantages such as controlled drug release properties, better drug stability and solubility, and lower toxicity [13]. Mohammed MA et al. in a review article on chitosan nanoparticles concluded that chitosan nanoparticles have the potential to be used as drug delivery system via the digestive tract, nasal and lungs because it is able to deliver drugs to specific locations while retaining the drug for extended drug absorption [14].
Histologically, the healing process is indicated by the density of collagen fibers produced by fibroblasts during the proliferation phase where this is stimulated by fibroblast growth factor-2 (fibroblast growth factor-2/FGF-2). Fibroblast growth factor-2 is a growth factor secreted by macrophages. FGF-2 expression increases shortly after injury, and reaches its peak on days 5–8. Fibroblasts regulate angiogenesis through the secretion of these growth factors [15]. Studies on rat periodontal ligament by Salamao M et al. showed higher expression of FGF-2 during the first day of experimental tooth movement, attributed to cellular events in the initial phase of the inflammatory response by orthodontic forces applied to the tooth. This growth factor is considered the most potent mitogen for periodontal cells and plays an important role in wound healing because it supports angiogenesis and induces the development of immature periodontal ligament cells [16].

Based on these considerations, the researchers wanted to further analyze the effectiveness of chitosan nanoparticles containing 0.7% tetracyclines by subgingival application as an adjunct therapy in the rat periodontitis models evaluated from clinical parameters and FGF-2.

**MATERIALS AND METHODS**

This research was an experimental laboratory research with a pretest posttest control group design. The manufacture of chitosan nanoparticles containing 0.7% tetracyclines was carried out at the Research Laboratory of the Faculty of Pharmacy, Universitas Sumatera Utara. *P. gingivalis* culture was carried out at the Microbiology Laboratory, Faculty of Medicine, Universitas Sumatera Utara. Treatments to experimental animal models and specimen samples was carried out at the Animal House, Faculty of Pharmacy, Universitas Sumatera Utara. Immunohistochemical examination will be carried out at the Histology Department, Faculty of Medicine, Universitas Sumatera Utara. The ethical feasibility of research was approved by the Research Ethics Commission of Universitas Sumatera Utara Faculty of Mathematics and Natural Sciences (No. 0250/KEPH-FMIPA/2021) and the Research Ethics Commission of Universitas Sumatera Utara Faculty of Medicine (No. 400/KEP/USU/2021).

Research samples were 33 rats induced with *Porphyromonas gingivalis*. Samples were put in cages which were cleaned daily, given food and drink and kept in a room with enough sunlight. They were divided into seven experimental groups:

1. **KA1**: scaling-root planing and application of chitosan nanoparticles with 0.7% tetracyclines were carried out every day, then clinical parameters and FGF-2 were examined on day 3 (rats were euthanized)
2. **KA2**: scaling-root planing and application of chitosan nanoparticles with 0.7% tetracyclines were carried out every day, then clinical parameters and FGF-2 were examined on day 8 (rats were euthanized)
3. **KBI**: scaling-root planing and application of chitosan nanoparticles with 0.7% tetracyclines were carried out once only, then clinical parameters and FGF-2 were examined on day 3 (rats were euthanized)
4. **KB2**: scaling-root planing and application of chitosan nanoparticles with 0.7% tetracyclines were carried out once only, then clinical parameters and FGF-2 were examined on day 8 (rats were euthanized)
5. **K+1**: scaling-root planing and application of placebo were carried out once only, then clinical parameters and FGF-2 were examined on day 3 (rats were euthanized)
6. **K+2**: scaling-root planing and application of placebo were carried out once only, then clinical parameters and FGF-2 were examined on day 8 (rats were euthanized)
7. **K**: on day 0, FGF-2 examination was carried out (rats were euthanized)

Inclusion criteria were adult male white *wistar* rats aged 8-12 w, body weight 200-250 grams, had lower central incisors and were systematically healthy. Exclusion criteria were rats that were not induced by periodontitis and were systemically diseased. Rats that died during the research were dropped out.

The research began by subculturing *P. gingivalis* bacteria on 5% *Brucella Bloodship* blood agar medium and incubating for 48-72 h in an anaerobic atmosphere at a temperature of 37 0C. Making a suspension of *P. gingivalis* begins by taking an inoculture tube containing 0.45% sodium chloride and then taking the resulting pure *P. gingivalis* colonies with a strength of 3-5 McFarlan. The bacterial suspension could be directly infected into animals. The experimental animals were adapted for 10 d and kept in cages placed in a room with sufficient air flow and light.

The periodontitis induction procedure began with administering intraperitoneal injection anesthesia to rats with ketamine 20 mg/kg BW to provide a sedation effect. Probing was carried out after the rats were anesthetized to measure the depth of the gingival sulcus in normal conditions (fig. 1). An incision was then made in the anterior area of the lower jaw teeth and a bone defect was created using a fine fissure bone bur. A ligature wire was tied around the cervix of the tooth to promote plaque retention and inflammation. Making experimental animal models of periodontitis was then continued by carrying out induction with *P. gingivalis* bacteria the following day in the gingival sulcus (fig. 2). Culture examination after day 3 d was carried out to ensure that the rats were infected with *P. gingivalis*. Periodontitis characterized by redness, gingival swelling, periodontal pockets and loss of gingival attachment appeared on the 6th day.

**Fig. 1: Probing sulcus depth before periodontitis induction**

**Fig. 2: Periodontitis model creation. A, B. Incision and creation of bone defects in the lower anterior teeth. C. Placement of a ligature wire in the cervical area of the tooth as deep as possible into the sulcusSD Injection of *P. gingivalis* bacteria**
Chitosan nanoparticles were made using the ionic gelation method of chitosan and sodium tripolyphosphate at room temperature. The stages are as follows (fig. 3) [13]

1. Chitosan (2 g) was dissolved in 1% acetic acid (50 ml) and stirred for 24 h on a magnetic stirrer at room temperature.
2. While continuing to stir, the pH was adjusted to 5.5 by adding 0.01 N NaOH (15 ml) to the chitosan mixture.
3. Tripolyphosphate/TPP (0.005 g) was dissolved separately in deionized water (0.5 ml), then the TPP solution was added dropwise to the chitosan solution and kept stirring at room temperature.
4. The mixture was homogenized using a homogenizer at a speed of 1 000 rpm for 1 min.

The Particle Size Analyzer (PSA) test was carried out to see the size distribution of the gel particles. Then a concentration of 0.7% tetracyclines (0.35 g [based on the composition of 0.7% tetracyclines gel based on chitosan hydrogel reported by Susanto C et al. in 2017] [9] dissolved in water was added to the chitosan nanoparticle gel. Stirring for 20 min was carried out again to obtain the final mixture of 0.7% tetracyclines based on chitosan nanoparticles [13].

Supragingival and subgingival scaling was performed on all experimental animals with an electric scaler. Chitosan nanoparticles containing 0.7% tetracyclines for the treatment group and placebo for the control (+) group was applied with a 1 cc syringe. The stages were that the tooth in question was isolated and then the tip of the syringe needle was inserted into each designated pocket without pressure to prevent tissue trauma. Gel was injected into the pocket until excess gel was seen coming out of the pocket and pooling around the neck of the tooth (fig. 4). Rats were not given food/drink for 1 h.

Specimen preparation began by euthanizing the experimental animals using the neck dislocation technique. Then the rat’s mandible was cut and the mandible was placed in a tube containing 10% formalin (fig. 5). The remaining rat tissue that was not taken for research purposes was immediately burned. The specimens were then decalcified in 10% EDTA at room temperature for 3-14 d, processed histologically and embedded in paraffin blocks (fig. 6) with the following stages:

1. The tissue pieces were placed in 10% buffered formalin solution for 18-24 h, then the tissue was placed in distilled water for 1 hour to remove the fixation solution.
2. The tissue pieces were placed in graded concentrations of alcohol, then the tissue was placed in an alcohol-xylol solution for 1 h and a pure xylol solution for 2 x 2 h.
3. The tissue pieces were placed in liquid paraffin for 2x2 h.

4. Tissue was embedded in solid paraffin which has a melting point of 56-58 °C. Microscopic preparations were obtained by thinly cutting paraffin blocks that had been stored in the refrigerator with a microtome 4 µm thick in the longitudinal direction.

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Fig. 5: Specimen preparation. A. Euthanasia of experimental animals using neck dislocation technique. B. Mandibular specimen. C. Soaking the specimen in 10% formalin

Fig. 6: Paraffin block

Each paraffin block was re-cut once for FGF-2 staining. Paraffin block samples that have been cut thinly (4 µm) are attached to a glass object using the sharp tip of a knife or tweezers and labeled. Thin strips were separated and flattened by placing them in warm water. Once it has expanded, transfer it to the object glass. Next, the object glass was placed on a heating device (hotplate) 50-60 °C. After the paraffin softened, the slide was dried and the tissue section was ready to be stained with primary antibody rabbit polyclonal FGF-2 (BioEnzy) (fig. 7). Immunohistochemical observation of FGF-2 expression was carried out under a binocular microscope. The data obtained from the research was qualitative data in the form of BOP clinical parameters, quantitative data in the form of Pocket Depth (PD) clinical parameters, and semi-quantitative data in the form of FGF-2 expression.

Qualitative data analysis used Fisher’s exact test and quantitative data analysis was tested for homogeneity and normality using the Shapiro-Wilk test. Next, to analyze the mean differences before and after application, the Wilcoxon test was carried out. Mean FGF-2 expression between groups was assessed using the Kruskal-Wallis test. If there was a significant difference, the data was tested further with Post Hoc Mann-Whitney.

Fig. 7: Rabbit polyclonal FGF-2 antibody (BioEnzy)

RESULTS AND DISCUSSION

During the research, 4 rats died so that the remaining samples at the end of the study were 29 rats.

Evaluation of clinical parameters after subgingival application of chitosan nanoparticles containing 0.7% tetracyclines

The clinical parameters evaluated were bleeds on probing (BOP) and Pocket Depth (PD) before and after subgingival application of chitosan nanoparticles containing 0.7% tetracyclines on day 3 and 8.

Table 1: Percentage of BOP before and after treatment in each group on day 3

<table>
<thead>
<tr>
<th>Group</th>
<th>BOP (%)</th>
<th>Before</th>
<th>After</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scaling + nanochitosan tetracyclines 0.7% everyday (n=5)</td>
<td>100</td>
<td>60</td>
<td>0.444</td>
<td></td>
</tr>
<tr>
<td>Scaling + nanochitosan tetracyclines 0.7% once only (n=4)</td>
<td>100</td>
<td>75</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>Scaling placebo (n=4)</td>
<td>100</td>
<td>75</td>
<td>1.000</td>
<td></td>
</tr>
</tbody>
</table>

*Fisher’s Exact test, significant p<0.05

Table 2: Percentage of BOP before and after treatment in each group on day 8
Table 1 and 2 show a decrease in the percentage of BOP in all groups after application of chitosan nanoparticles containing 0.7% tetracyclines on day 3 and 8. However, a significant decrease was only found in the application group of chitosan nanoparticles containing 0.7% tetracyclines everyday on day 8.

Table 3 and 4 show a decrease in p value in all groups on day 3 and 8. However, a significant decrease was only found in the application group of chitosan nanoparticles containing 0.7% tetracyclines, either everyday or once.

Evaluation of FGF-2 Expression After Subgingival Application of Chitosan Nanoparticles Containing 0.7% Tetracyclines

The expression of FGF-2 before and after subgingival application of chitosan nanoparticles containing 0.7% tetracyclines on day 3 and 8 can be seen in the following graph.
Fig. 8 shows an increase in FGF-2 expression on day 3 of each group and a decrease in FGF-2 expression on day 8 of each group. The samples used on day 0, 3, and 8 were from different rats.

Table 5 shows that there is a significant difference in the mean FGF-2 expression in all groups on day 3. In contrast, table 6 shows that no significant differences were observed on day 8. To further evaluate the differences in the mean FGF-2 expression on day 3 after treatment, the results of the post hoc test are presented in table 7.

Table 7 shows a significant expression of FGF-2 on the 3rd day after application of chitosan nanoparticles containing 0.7% tetracyclines every day compared to a single application, but it was not significant compared to placebo. The expression of FGF-2 on daily application of chitosan nanoparticles containing 0.7% tetracyclines was also not significant compared to placebo application. The immunohistochemical image is presented in fig 9.

Table 7. Comparison of FGF-2 expression between each group on day 3 after treatment.

<table>
<thead>
<tr>
<th>Group</th>
<th>FGF-2 (±SD)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scaling+nano-chitosan tetracyclines 0.7% everyday (n=5)</td>
<td>2.40±0.54</td>
<td>0.395*</td>
</tr>
<tr>
<td>Scaling+nano-chitosan tetracyclines 0.7% once only (n=4)</td>
<td>2.00±0.00</td>
<td></td>
</tr>
<tr>
<td>Scaling+placebo (n=4)</td>
<td>2.25±0.50</td>
<td></td>
</tr>
</tbody>
</table>

*Kruskal-Wallis test, *significant p<0.05

Fig. 9: Immunohistochemical image for FGF-2 expression in the application group of chitosan nanoparticles containing 0.7% tetracyclines every day for 3 d. The proportion score is calculated based on the percentage of the number of fibroblast cells (purple color) that express FGF-2 (brown color) and the intensity score is assessed based on the concentration of brown color expressed by fibroblast cells. Observations were made under 400 times magnification.

Chitosan as a drug delivery biopolymer used in this research has biodegradable properties, is non-toxic to mammalian cells, and has broad antimicrobial activity against both gram-positive and gram-negative bacteria. The antibacterial activity resulting from nanoparticle suspensions is higher than chitosan solutions because the smaller the particle size, the greater the surface area and reactivity of chitosan, thereby increasing the interaction between charges on the bacterial surface and its antimicrobial effect [19].

Evaluation of clinical parameters after subgingival application of chitosan nanoparticles containing 0.7% tetracyclines

Research conducted on a rat periodontitis model to assess the effectiveness of chitosan nanoparticles containing 0.7% tetracyclines given every day showed a significant decrease in the percentage of BOP on day 8. A systematic review conducted by Matesanz-pe P et al. regarding the effect of antimicrobials as a support for subgingival debridement in the treatment of chronic periodontitis showed that there was a significant difference in changes in BOP values statistically compared to the control group [17]. An article by the American Academy of Periodontology in 2021 stated that BOP is a diagnostic tool to evaluate periodontal disease status where the absence of BOP is considered as a highly specific (88%) indication of healthy periodontal and the continued absence of BOP during the maintenance phase is considered as an indicator of stable periodontal health [20].

Apart from the decrease in BOP, a significant decrease in mean PD on day 3 and 8 was also found in the chitosan nanoparticle application group containing 0.7% tetracyclines which was given every day or just once and was not significant in the control group. Matesanz-pe P et al. in a meta-analysis of five studies evaluating the effectiveness of tetracyclines fibers also showed a change in the mean PD [17]. A systematic review by Rovai ES et al. showed that using local antimicrobials as an adjunct to non-surgical periodontal therapy in patients with diabetes can improve PD and CAL [21]. Another systematic review of smokers with chronic periodontitis also showed that the use of supportive antibiotics can increase PD reduction and CAL increase after SRP over a 6 mo period [22].
The significant improvement in gingival inflammation, gingival bleeding and pocket depth in this study can be attributed to the effectiveness of tetracyclines in inhibiting the growth of periodontal pathogens [5]. In addition, chitosan is also bactericidal or bacteriostatic. This property is mediated by the electrostatic interaction between the protonated NH3+ group and negative residues on the surface of the cell membrane. This electrostatic interaction causes changes in the permeability of the cell membrane walls, thereby triggering an internal osmotic imbalance and consequently inhibiting the growth of microorganisms and the hydrolysis of peptidoglycan in the walls of microorganisms [23]. Li et al. showed a close correlation between the relative expression of periodontal bacteria P. gingivalis and the PD where the elimination of these pathogens can prevent the development of periodontitis [24].

Areas with PD>5 mm treated with SRP and local antibiotics may have greater benefits of PD reduction and CAL improvement compared with SRP alone [22]. Deeper periodontal pockets tend to show greater clinical improvement after non-surgical periodontal treatment than pockets shallow ones [25].

Scaling-root planing itself is very effective in reducing inflammation and pocket depth, but bacterial recolonization in periodontal pockets can still occur after the scaling-root planing procedure. The effectiveness of scaling-root planing will decrease with increasing pocket depth, root concavity, restoration contour, furcation involvement, and irregularity of the root surface and dentin tubules [26]. The administration of chitosan nanoparticles containing 0.7% tetracyclines as an adjunct for scaling-root planing is expected to provide clinical improvements in case treatment. periodontitis with these complicating factors.

**Evaluation of FGF-2 expression after subgingival application of chitosan nanoparticles containing 0.7% tetracyclines**

Subgingival application of chitosan nanoparticles containing 0.7% tetracyclines in a rat periodontitis model showed higher FGF-2 expression in the day 3 sample group and lower FGF-2 expression in the day 8 sample group. The FGF-2 level was higher on day 3 in this study because FGF-2 plays a role in inducing the angiogenesis process and is involved in signal transmission between the epithelium and connective tissue [28]. The lower FGF-2 level on day 8 is thought to occur due to tissue repair as seen from a decrease in BOP and PD.

Research by Wilson on rat periodontitis models showed that fibroblast cells count in the periodontal ligament appeared to be greater in the group given a combination of SRP therapy accompanied by the application of 0.7% tetracyclines based on chitosan hydrogel for 7 d compared to the control group [27]. The increase in the number of fibroblast cells indicates a periodontal regeneration process which is induced by FGF-2 in the early phase of cell proliferation [26].

This study shows that the expression of FGF-2 after application of chitosan nanoparticles containing 0.7% tetracyclines every day is not significant in the control group. The non-significant difference in FGF-2 expression between the treatment group and the control group in this study may be caused by the degree of inflammation in the mild periodontitis model and shallow pockets so that no statistical difference was seen. In addition, FGF-2 expression may need to be supported by examining other parameters such as the number of fibroblast cells, collagen fiber density, or collagenase expression which indicate the healing and regeneration process of periodontal tissue.

Franco Ej et al. in their research compared the effectiveness of the combination of SRP-photodynamic therapy with SRP alone and the result was that there was FGF-2 expression which was three times higher than the control group. FGF-2, which is expressed in various tissues, has effects on various types of cell. Protein encoded by this gene promotes the proliferation of fibroblasts and osteoblasts and has a very strong induction effect on angiogenesis [29].

Research conducted by Nagayasu-Tanaka T et al. on beagle dogs to evaluate the mechanism of FGF-2 in periodontal regeneration showed that the regeneration process was the same in the control and FGF-2 groups. However, compared to the control group, the mass of fibroblastic cell tissue formed was wider throughout the root surface in the FGF-2 group and the height of the connective tissue formed increased more significantly with the administration of FGF-2. The facilitation mechanisms of FGF-2 in periodontal regeneration demonstrated in vivo include: (1) FGF-2 accelerates and increases the proliferation of fibroblastic cells originating from the bone marrow and periodontal ligament for the formation of new tissue, (2) FGF-2 increases angiogenesis, and (3) FGF-2 triggers the expression of bone morphogenetic protein/BMP-2 to facilitate osteoelastic differentiation and bone formation. This suggests that during the early stages of periodontal tissue regeneration, FGF-2 increases the number of fibroblastic cells and supports angiogenesis [30].

From this description, it can be seen that the daily application of chitosan nanoparticles containing 0.7% tetracyclines has better advantages compared to a single application. Therefore, in future studies in periodontitis patients who do not allow daily application, it is recommended to use a periodontal dressing to keep the tetracyclines gel lasting longer in the pocket.

**CONCLUSION**

The effectiveness of the application of chitosan nanoparticles containing 0.7% tetracyclines as an initial therapy support for FGF-2 expression showed a higher amount of FGF-2 expression on day 3 and a lower amount on day 8. However, research still needs to be carried out to evaluate the effect of application of chitosan nanoparticles alone and a mixture of chitosan nanoparticles containing 0.7% tetracyclines on periodontitis patients who have indications for antibiotic therapy as well as the curvicular concentration that can be achieved and the length of time this concentration can last in the pocket.

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**AUTHORS CONTRIBUTIONS**

Silvia-Concept, writing original draft preparation and revision manuscript, data design and analysis, performed the experiments; Irma Ervina-Supervision and visualization, revision of manuscript; Martina Amalia-Supervision and visualization, revision of manuscript; Erdi Elfeni-Naution-Revision manuscript, data design and performed the experiments; Harry Agusnar-Supervision and visualization, revision of manuscript; Pitu Wulandari-Supervision and visualization, revision of manuscript; Rini Octavia-Revision and visualization, revision of manuscript.

**CONFLICT OF INTERESTS**

There is no conflict of interest regarding the publications of this paper.

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