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Original Article

EFFECTIVENESS OF CHITOSAN-LOADED TETRACYCLINE IN PERIODONTITIS INDUCED RATS

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ABSTRACT

Objective: The primary treatments for periodontal disease are scaling and Root Surface Debridement (SRSD), which involves eliminating bacteria plaque and achieving a smooth root surface area. Tetracycline helps increase attachment and enhances the regeneration of alveolar bone and tissue. Chitosan is an adequate system for local drug preparation in periodontal pockets. The purpose of this study was to evaluate the effectiveness of chitosan tetracycline hydrogel in pocket depth and collagen density in periodontal ligaments in animal models.

Methods: Three groups of Wistar rats were categorized. The first group treatment was SRSD with tetracycline 0.7% based chitosan hydrogel for seven days; the second group was SRSD with an application of tetracycline 0.7% based chitosan hydrogel on the first day, and the third group treatment was SRSD only. After treatment, pocket depth was evaluated at the baseline and on the 14th day, along with the examination of collagen tissue density in the periodontal ligament, which appeared as blue fibers with Masson's trichrome staining, was seen and compared to other groups.

Results: The most significant change in the pocket depth was observed in this study, along with improved collagen tissue density, was found in the group of SRSD with an application of tetracycline 0.7% based chitosan hydrogel for seven days, and this result was statistically significant ($p<0.05$) compared to other groups.

Conclusion: Tetracycline 0.7% based chitosan hydrogel for seven days shows a better result in decreasing pocket depth and increasing collagen tissue density in periodontal ligaments.

Keywords: Periodontal disease, Tetracycline, Hydrogel, Chitosan, Collagen

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INTRODUCTION

Scaling-Root Surface Debridement (SRSD) is one of the most commonly used procedures for treating periodontal disease and has been used as a gold standard. Clinical effects show that SRSD decreases pocket depth and attachment loss. Mechanical treatment is effective in reducing levels of subgingival microorganisms, but not all pathogens can be eliminated. Haffajee reported that SRSD itself has limited effects on some pathogenic species, in the case of tissue invasion by periodontal pathogens, such as Porphyromonas gingivalis or Aggregatibacter actinomycetemcomitans, where mechanical therapy is not enough to remove bacteria from the pocket. Therefore, apart from careful treatment, rapid progression from loss of attachment and resorption of alveolar bone can occur. In such cases, antimicrobial adjunct therapy can be effective [1-3].

Using antibiotics locally to support mechanical therapy provides a better response to treatment, especially in deep-pocket conditions. Giving antibiotics locally can be done in dealing with infections localized to the periodontal tissue. Tetracyclines are either produced naturally in certain Streptomyces species or semi-synthetically. The bacteriostatic properties of Tetracyclines make them effective against bacteria that multiply rapidly. Gordon *et al.* have shown that tetracycline at low concentrations (2-4 µg/ml) is effective against many periodontal pathogens. Tetracycline is commonly used because it has broad-spectrum properties, can penetrate bone tissue, and also inhibits the matrix metalloproteinases and collagenases released by host immune cells in response to microorganisms [2, 4].

Collagen is crucial at every stage of the wound-healing process. The primary function of collagen is as a support in connective tissue. At the beginning of the wound healing process, type III collagen is the first visible collagen, replaced by type I collagen as granulation and remodeling begin. Matrix metalloproteinases that increase in periodontitis can cause collagen damage in periodontal tissue, including the periodontal ligament area, where many collagen fibers support this tissue [5-7].

Sachdeva, in the clinical study, stated that the use of tetracycline locally combined with the scaling-root surface debridement procedure in cases of periodontitis can reduce pocket depth, gingival index, and plaque index. The biocompatibility of the use of tetracycline has been investigated by Maduratna. In the form of tetracycline gel with a concentration of 0.7% can be well received by the tissue. Mohammed M, in his research, stated that doxycycline gel was effective against periodontal pathogenic bacteria [7, 8].

Chitosan is a drug-delivered system; it has biodegradation, biocompatible, non-toxic, and non-toxic properties; this material has been widely used in various drug transportation systems; compared to other transportation systems, chitosan nanoparticles have special features. Popa L, Ghica M, and Elena C, in their research on the use of chitosan as a gel raw material, stated that chitosan is an adequate system for releasing drugs locally in periodontal pockets, this material can remain in the pocket, and the release of antimicrobial agents in the crevicular fluid can be controlled. The release of Tetracycline gel was tested by Popa using either 1% or 3% chitosan concentrations. George *et al.* in 2006 investigated the toxicological properties of chitosan, claiming that chitosan is a biodegradable and non-toxic hydrophilic polymer [9, 10].

Research by Susanto shows that chitosan can be used as a topical drug conductor where chitosan material can release tetracycline in chitosan gel. The average diameter of inhibition zones of all tetracycline gels is more than 27 mm, indicating that the antibacterial activity of 1%, 0.7%, and 0.5% chitosan-based tetracycline gel is very strong. Chitosan-based tetracycline 1% gel has the greatest antibacterial activity because it shows the largest inhibitory zone diameter compared to tetracycline gel 0.7%, 0.5% based on chitosan and

chitosan gel without tetracycline. Andrew *et al.*, in their research, showed non-toxic properties of tetracycline, which is seen in 0.5%, 0.7%, and 1% tetracycline gel in 4% chitosan hydrogel, which does not refer to the cytotoxic properties of fibroblast cells. This study aims to assess the efficacy of chitosan hydrogel 0,7% tetracycline when applied locally, based on reducing the pocket depth and increasing collagen tissue density in animal models [11, 12].

MATERIALS AND METHODS

The sample of this study was taken from rats induced with bacteria from male Wistar rats aged 8-12 w with a weight of 200-250 grams and had a healthy body condition. The study sample was divided into three groups, group A treatment was SRSD with an application of tetracycline 0.7% based chitosan hydrogel for seven days, Group B treatment was SRSD with an application of tetracycline 0.7% based chitosan hydrogel only on the first day and Group C as a control group was given SRSD treatment only. The pocket depth was evaluated by counting how deep it was from the gingival margin to the apical of the pocket on the central incisor of rats before SRSD and after 14 d in three groups. Ethical clearance information is obtained by submitting a research proposal to the Research Ethics Commission of the Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, and the Faculty of Medicine, Universitas Sumatera Utara (649/TGL/KEPK FK USU-RSUP HAM/2019).

Bacteria isolate sub-culture

The subculture of the bacterial isolate Prophyromonas gingivalis began with cultivation on a 5% Brucella blood ship primary culture medium with a four-quadrant strick, then incubated for 48-72 h in an anaerobic atmosphere at 370 °C.

A pure colony of Prophyromonas gingivalis and an in-culture tube with 0.45% sodium chloride are used to create the suspension. Afterward, the bacterial suspension can be directly infected with test animals.

Creating periodontitis rats' model

The procedure begins with Intraperitoneal injection anaesthetics performed in rats with ketamine 20 mg/kg BW and xylazine 5 mg/kg BW to provide a sedation effect. Surgically made an incision in the vestibule of the left and right incisors on the lower jaw, full-thickness fibers are used to expose the alveolar bone to the vestibular region. The alveolar bone defects in the vestibular region are exposed by using bur carbide. Defects were made in the vestibular area by reducing the alveolar bone height by 1.5 mm and 3 mm in width using a carbide bur. The flap is positioned to its original position and sewn using silk 5-0 and stitched with the horizontal mattress technique, and rats are given a high carbohydrate diet. The bacterial suspension was induced in the gingival sulcus region one week later. The stitches are taken off after three days of induction, and swabs are taken in the gingival sulcus area for a microbiological culture analysis than the rats have been infected with Prophyromonas gingivalis bacteria.

Preparation of tetracycline 0,7% based chitosan hydrogel

Tetracycline gel 0,7% based on chitosan hydrogel was prepared using the manufacturing method reported by Susanto in 2016.11 Sterilization of all tools used to produce 0.7% tetracycline gel based on chitosan hydrogel is done in an oven at 170 °C for 1 h. In a laminar air flow cabinet, tetracycline gel is made aseptically. After mixing chitosan with 1% lactic acid, it was poured into a stamper to create a 4% chitosan hydrogel. Tetracycline was added and stirred slowly until homogeneous and formed 0.7% tetracycline based on chitosan hydrogel.

Histologic preparations

On the 14th d, the rats were euthanized by inhaling ether and then their mandibles were taken. The specimens were rinsed using a saline solution and then neutralized using 10% buffered formalin. The specimens were split into smaller sizes and dehydrated by immersing them in alcohol with a grade of 70%, 80%, 95%, 100% for 2 h. Alcohol was used to clean the specimens by immersing them in Xylol solution twice and soaking each one for 1.5 h. The tissue block was filled with paraffin by immersing the specimens in liquid paraffin three times for two hours each. The process of sectioning the tissue blocks using a 5 m microtome knife in a buccal-lingual direction and placing it on a slide. The slides containing paraffin were immersed in xylol solution 2 times, alcohol 2 times for 1 min, 95% alcohol each for 1 min, and iodine solution for 10 min, then immersed 4 times in running water to be immersed in Masson trichrome solution for 10 min.

Histological observations were made using a light microscope and an Olympus camera with 400 x magnification. Collagen fiber density measurements were performed by calculating the density score in the histological preparations using 5 visuals. The measured periodontal ligament collagen density is located beneath the fused epithelium to the apical. Collagen fibers are present in connective tissue and are measured in percentages based on the color fraction produced by each preparation using an image processing software program (Image J). Measurements were made based on the observation time before treatment and on the 14th d after treatment and compared the average between the control group and the treatment group at each observation time.

Data analysis

The study data will be examined by analyzing clinical parameters before and after treatment along with the density of periodontal ligament collagen after treatment, using a ratio scale. Data analysis is found to be normal, then the Anova test is carried out, conversely, if the data is not normal, the Kruskal-Wallis and Wilcoxon tests are carried out.

RESULTS AND DISCUSSION

This research was conducted to test the role of tetracycline 0,7% based on chitosan hydrogel in the periodontitis model. The parameters in this study are pocket depth and collagen tissue density in periodontal ligaments. Twenty-seven Wistar rats were used in these studies that were induced by Porphyromonas gingivalis bacteria.

Pocket depth

Test results in probing Pocket Depth (PPD) on the baseline and 14 d are postoperative that consist of treatment SRSD with or without tetracycline 0.7% based chitosan hydrogel is shown in table 1 and table 2.

Table 1: Differences of mean value in pocket depthon the baseline and 14 d postoperative in each group

Group	PPD mean- value ($\bar{x}$±SD)		*p-value*
	Baseline	14 d post-treatment	
SRSD+LTCH for 7 d	1.72±0.36	0.27±0.36	0.006*
SRSD+LTCH on 1st day	1.50±0.25	0.97±0.19	0.01*
SRSD	1.77±0.26	1.72±0.34	0.57

**Kruskal-Wallis* test, PPD=probing pocket depth, SRSD=scaling and root surface debridement, LTCH= local tetracycline 0.7% based chitosan hydrogel, significant *P<0.05.*

Based on table 1 above, there was a reduction in pocket depth in each group after 14 d of SRSD. The reduction in pocket depth was statistically significant (p<0.05) only in the group given application of LTCH and was not significant in the SRSD group alone.

The density of collagen tissue in periodontal ligament

The test results for differences in the percentage of collagen density of periodontal ligaments after 14 d of SRSD with and without the application of tetracycline are shown in table 3.

Table 2: A comparative test of change in pocket depth after 14 d SRSD in each group

Group	PPD changes-($\bar{x}$±SD)	*p-value*
SRSD+LTCH for 7 d	1.45±0.35	
SRSD+LTCH on 1st day	0.53±0.31	0.000*
SRSD	0.05±0.18	

*Kruskal-Wallis test, PPD=probing pocket depth, SRSD=scaling and root surface debridement, LTCH= local tetracycline 0.7% based chitosan hydrogel, significant P<0.05. Based on table 2, the mean decrease in pocket depth after 14 d SRSD was greater in the tetracycline application group for seven days than in the other groups and was statistically significant (p<0.05).

Table 3: Test differences in the percentage of periodontal collagen ligament density after 14 d in each group

Group	Percentage of collagen density, (□±SD)	*p-value*
SRSD+LTCH for 7 d	68.64±7.82	
SRSD+LTCH on 1st day	46.93±3.61	0.000*
SRSD	43.7±6.41	

*ANOVA test, SRSD=scaling and root surface debridement, LTCH= local tetracycline 0.7% based chitosan hydrogel, significant *P<0.05.*

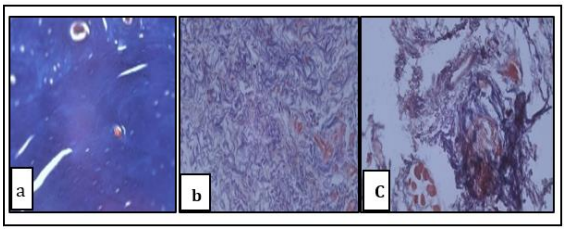

Fig. 1: Histologic picture of collagen density in periodontal ligaments by Masson's trichrome staining with one sample from each group. Collagen tissue density in the periodontal ligament appeared as blue fibers. (A) Histological picture of collagen density in the group A treatment. (B) Histological picture of collagen density in the group B treatment. (C) Histological features of collagen density in the control group. Shooting is done under an Olympus light microscope at 400 x magnification

Three groups with Masson's trichrome staining under an Olympus light microscope. The appearance of collagen is seen as blue fibers and is expressed in percentage based on the color fraction produced by each preparation using an image processing software program. The dense collagen tissue was seen in the tetracycline application group for seven days compared to the other groups.

This study showed a reduction in pocket depth in the group given 0.7% tetracycline antibiotic based on chitosan hydrogel, which appeared to be statistically significant (p<0.05) compared to only SRSD alone. The decrease in pocket depth after 14 d was greater in the tetracycline antibiotic group for seven days than in the other groups. Tetracycline based on chitosan hydrogel has been one of the most investigated antimicrobial agents considering its local release and its important property of featuring substantivity by its affinity with the radicular surface; this drug has been demonstrated to be effective against the periodontopathogenic microbiota and inhibiting collagenase.

Kafle examined tetracycline fiber by the local administration to treat periodontitis with a subject of 30 people between the ages of 35-45 y with an average pocket depth of 5-8 mm in the contralateral region. Kafle found a significant decrease in pocket depth in month two and month three. According to Babrawala, natural chitosan 1% could be used as a local drug delivery system in addition to SRSD in non-surgical periodontal therapy. Chitosan can inhibit the growth of microorganisms, have anti-inflammatory properties, and accelerate wound healing. His research found that chitosan with a local antibiotic mixture can reduce pocket depth, gingival index, and bleeding index [13, 14].

In another study by Sadaf, 30 patients diagnosed with periodontitis in the age group 35 to 55 were selected who had a clinically sized periodontal pocket of 4-7 mm and showed radiographic evidence of moderate bone loss. Plaque index, gingival bleeding index, and pocket depth were measured at baseline in 15, 30, 60, and 90 d. Tetracycline therapy significantly reduced pocket depth (p<0.001) in 0 and 3 mo, than SRSD alone. Microbial analysis showed a significant reduction in Porphyromonas gingivalis and Prevotella intermedia, although there was no significant* reduction in Actinobacillus actinomycetemcomitans [15].

Fernandes' study showed that subgingival irrigation with tetracycline hydrochloride was an effective adjunctive treatment for periodontitis induced in 60 rats that were divided into two groups according to the following treatment, subgingival irrigation with 1 ml of saline and subgingival irrigation with 1 ml of tetracycline HCL (50 mg/ml). Histometric analysis, at 7, 15, and 30 d, Group T (0.72±0.05 mm2, 0.57±0.14 mm2, 0.62±0.07 mm2), showed less bone loss (p<0.05) than Group C (1.35±0.25 mm2; 1.40±0.31 mm2; 1.29±0.27 mm2), respectively [16].

Compared to the first and control groups, the group given 0.7% tetracycline-based chitosan hydrogel for seven days showed more collagen formation, which was statistically significant (p<0.05). Research Gusti examined the use of 0.7% tetracycline HCl in the gingival sulcus of rats with periodontitis and found a significant increase in collagen fibers in periodontal ligaments on the 10th d of observation by administering tetracycline HCl 0.7% once daily for eight days compared to the control group that was only given a placebo gel.

Darmastuti carried out another study with a total of 12 rabbits subjected to 75 mg/ml tetracycline HCl application and irrigation after curettage. Three rabbits were decapitated on days 3, 5, 7, and 10 for histological preparations. with Trichrome Mallory staining,

the research results obtained from the calculation of the color fraction of collagen fiber density showed that there were significant differences in collagen fiber density between the treatment and control groups on days 3, 5, 7, and 10 [17].

Gomes carried out another study on male Wistar rats, where both the control and treatment groups were given doxycycline for 7 and 14 d. Histological analysis using microsite red staining showed an increase in type 1 collagen in the doxycycline treatment group after seven days. At the dose of doxycycline 10 mg in the treatment group compared to the control group, a significant increase in collagen fibers on day 7 and day 14 in the treatment group receiving doxycycline 10 mg compared to the control group showed better bone formation. Although no percentage difference in collagen fibers was seen between the doxycycline 25 mg and control groups, the doxycycline 25 mg group showed a significant amount of type 1 collagen fibers [18].

Pang *et al.* researched the effects of chitosan on human periodontal ligaments *in vitro*. They found that chitosan can increase the synthesis of type 1 collagen and facilitate the differentiation of osteogenic cells, thereby accelerating tissue and bone growth [19].

These results show a negative correlation between clinical parameters, i.e. pocket depth to the density of collagen tissue in the periodontal ligament in each group but not statistically significant ($p>0.05$). Fatimah *et al.* Conducted a study of 72 subjects divided into three groups: the healthy patient's group, gingivitis, and generalized periodontitis. The study results showed a negative correlation between pocket depth with type 1 collagen but were not statistically significant [20].

In this study, the administration of 0.7% tetracycline antibiotic based on chitosan hydrogel given for seven days accompanied by SRSD showed a decrease in the number of modified papilla bleeding index, pocket depth, and a significant increase in collagen density in periodontal ligaments. Scaling-root surface debridement is the gold standard in periodontal treatment because it can have beneficial clinical effects during periodontal healing even without additional therapy. The SRSD procedure provides the effect of antimicrobial therapy by removing bacteria mechanically. SRSD is also able to get rid of endotoxins, reduce the number of bacteria, and suppress inflammation. Cleaning periodontal pathogens and their products with SRSD is good but not optimal. Some parts cannot be accessed by SRSD devices, such as periodontopathogenic bacteria in the dentine tubules, gingiva, and cementum, still lagging. Olsvik suggested that SRSD alone was not enough to eliminate the Aa and Pg bacteria. Provision of systemic antimicrobials orally or locally is recommended to improve the results of SRSD therapy. Gordon stated that tetracycline administration was needed to suppress bacterial growth in pockets [21-24].

Therapy of destructive periodontal disease will be effective if antibiotics can penetrate the area of infection into the periodontal pocket. Tetracycline applied subgingivally to the periodontal pocket will directly enter the subgingival area so that side effects to other areas are minimal. Tetracycline can increase reattachment and regeneration by increasing the attachment of fibroblasts that synthesize collagen on the root surface and inhibit collagenase activity. This is because tetracycline can inhibit collagenase enzymes produced by bacteria, so tetracycline is referred to as anti-collagenolytic antibiotics. This property benefits periodontal tissue because it inhibits damage that occurs in periodontal disease [22, 25-27].

CONCLUSION

Within the limits of the present study, it was possible to conclude that there was a decrease in pocket depth after SRSD treatment and a higher density of collagen tissue with an application of tetracycline 0.7% based chitosan hydrogel in rat periodontitis model. Further research is needed on the stability and resistance to tetracycline 0.7% based on chitosan hydrogel for subgingival application and the effective application of chitosan hydrogel-based tetracycline in human subjects.

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Nil

AUTHORS CONTRIBUTIONS

Winda Dwi Astuti-Conception, writing original draft preparation and revision manuscript, data design and analysis, performed the experiments; Irma Ervina-Supervision and visualization, revision of manuscript; Wilson-Writing original draft preparation and revision manuscript, data design and performed the experiments; Martina Amalia-Supervision and visualization, revision of manuscript; Armia Syahputra-Supervision and visualization, revision of manuscript; Rini Octavia Nasution-Supervision and visualization, revision of manuscript; Harry Agusnar-Supervision and visualization, revision of manuscript; Syafruddin Ilyas-Supervision and visualization, revision of manuscript.

CONFLICTS OF INTERESTS

Declared none

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