

Periodontal healing after Root conditioning using TTC-HCl or citric acid: A histological study in rats

Reparo periodontal após condicionamento radicular com TTC-HCL ou Ácido cítrico: Estudo histológico em ratos

Lucilene Hernandes RICARDO

Professora – Biopatologia Bucal – Pesquisadora Visitante – Programa de Pós-Graduação em Odontologia – Faculdade de Odontologia – Universidade de Taubaté – UNITAU – Taubaté – SP – Brasil

Ivan BALDUCCI

Professor Assistente – Disciplina de Bioestatística e Metodologia Científica – Departamento de Odontologia Social e Clínica Infantil da Faculdade de Odontologia de São José dos Campos – UNESP – São José dos Campos – SP - Brasil

Luana Marotta Reis VASCONCELOS

Doutoranda – Programa de Pós-Graduação – Biopatologia Bucal da Faculdade de Odontologia de São José dos Campos – UNESP – São José dos Campos – SP - Brasil

Yasmin Rodarte CARVALHO

Professor Adjunto – Disciplina de Patologia Bucal – Departamento de Biociências e Diagnóstico Bucal da Faculdade de Odontologia de São José dos Campos – UNESP – São José dos Campos – SP - Brasil

ABSTRACT

Tissue healing after periodontal therapy includes epithelial and connective tissue repair by producing biocompatible root surface. The objective of this study was to investigate whether etching solutions such as Tetracycline-hydrochloride (TTC-HCl) or citric acid might enhance the healing after periodontal therapy in rats. Periodontal defects were induced on the left maxillary 2nd molar in 36 Wistar rats. The periodontal attachment was reduced using a ligature model. The experimental sites were mechanically treated and delineated by blindly preparing a notch at the most apical aspect of the pocket using a Hedstroen endodontic file. The experimental sites were irrigated with a 10% tetracycline hydrochloride solution, an 8% citric acid solution, or a 0,9% NaCl solution (control) thus forming 3 groups of 12 animals each. The animals were euthanized at 3, 7 and 21 days post-treatment when block sections of the experimental sites were collected for histometric analysis. The histological observations describe normal wound maturation without regeneration in the defective site. Using a grid point counting method and two-way analysis of variance, it was shown that tissue fill in the notch did not differ significantly between sites irrigated with the acid solutions. However, tissue fill in these sites was significantly smaller than in the control sites. The results also showed that the area occupied by inflamed connective tissue was wider in the control compared to sites treated with the acid solutions. Based on these results it was concluded that irrigation with an acid solution could enhance healing following periodontal therapy

KEY WORDS

Tetracycline; dental pulp cavity; citric acid; periodontal repair

INTRODUCTION

The main objective of periodontal therapy includes tissue repair by producing biocompatible root surface. Based on the concept that periodontal disease begins by the interaction of the microbiological challenge and the host response against this injury, the healing of periodontal tissues may depend on the type of planned periodontal therapy. Some authors believe that conservative therapy, which includes oral hygiene

instructions and root planning, is enough to transform the root surface into a biocompatible one¹. However, other authors consider that the characteristics of the periodontitis-affected root surface facilitate bacterial colonization and make the elimination of them and their respective toxins difficult¹⁴.

The use of root surface chemical conditioner agents, which act in association with mechanical root planning, has been the objective of many studies^{13,23,25}. Among the studied agents, tetracycline and citric acid

have been shown as efficient for root conditioning and for removing the smear layer produced by mechanical instrumentation^{10,27}.

Tetracyclines comprise a broad-spectrum antimicrobial agent, which is effective against many species of periodontal pathogens. Besides its antimicrobial effectiveness, this group of drugs has other special properties used in the management of periodontal disease. Included among these are its anti-inflammatory action, collagenase inhibition, bone resorption inhibition and its ability to improve fibroblast attachment. Tetracyclines are still used in association with bone grafting and as conditioner agents for the root surface, and enhance periodontal tissue regeneration¹⁹.

The substantivity of tetracyclines is another important property for periodontal therapy. This property allows the substance to affix to a substrate and realize slow release, which is important in maintaining the antimicrobial and nonantimicrobial properties acting on the periodontal tissues for an extended time, when the drug is locally applied after periodontal instrumentation^{7,21,22}.

Even though some studies have demonstrated that the use of citric acid as root conditioner agent is similar to tetracycline^{4,8,10}, this substance has no antimicrobial or anti-inflammatory properties^{15,20}. When *in vitro* and *in vivo* studies that use tetracycline or citric acid as a root conditioner agent are compared, contradictory results are found, because it is difficult to control all the variables involved in an *in vivo* study¹⁶. However, in *in vitro* studies, it is impossible to observe all the interactions included in tissue healing. The use of animal models, in periodontal studies, with local root surface conditioners, and the histological analyses of the tissue repair can be an alternative to evaluate the effectiveness of the therapy. The aim of this study was to analyze the periodontal repair by comparing periodontal instrumentation associated with local use of 10% tetracycline, 8% citric acid or 0.9% NaCl in the treatment of induced periodontitis in rats.

MATERIAL AND METHODS

Animals

Thirty-six adult male Wistar rats, which were 10 weeks old and weighed 250g, were used in this study. The animals were maintained in cages, five animals in each, at room temperature, fed with water and food, provided by the São José dos Campos School of Den-

tistry - UNESP. The research protocol of this study was approved by Ethical Committee of Research of this institution and followed the guidelines for proper handling of laboratory animals from the Brazilian Association for Laboratory Animal Science (COBEA).

PERIODONTITIS INDUCTION

The animals were weighed before anesthesia. The solution was formed with Xylazine Chloride as a muscular relaxant plus Ketamine base as a general anesthesia, in a 1: 0.5ml proportion, with a 0.1ml dosage per 100g of rat body weight. A cotton ligature was placed around the cervix of the left maxillary second molar to induce periodontitis. The ligature was knotted on the buccal area of the tooth resulting in a sub gingival position in the proximal and in the palatal area and in a supragingival position on the buccal side. It was maintained there for five weeks, in accordance with Karimbuxs's study (Karimbuxs et al. 1998). During the induction period, the animals were examined once a week to check for ligature stability inside the gingival sulcus.

Periodontal Therapies

After five weeks, the ligatures were removed and the teeth were instrumented to remove calculus and bacterial plaque. Afterwards, the bottom of the pocket was marked with a notch. This notch was made by inserting a Hedstroen nº 20 endodontic file into the proximal space, in the buccal-lingual direction and then 20 movements were made to remove dentin in this region. After this the animals were separated into three groups: Group I = scaling associated with 1ml of 10% TCC-HCl irrigation (12 animals); Group II = scaling associated with 1ml of 8% citric acid irrigation (12 animals); Group III = scaling associated with 1ml of 0.9% NaCl irrigation (12 animals) as a control group. Rats included in each experimental group were sacrificed on the third, seventh or 21st days after the treatment. Following this, the maxilla was removed and fixed in a 10% formol solution for at least 48 hours.

Histological Analysis

The fragments were decalcified in a 20% formic acid aqueous solution. Following the decalcification they were processed to obtain 6µ thick serial sections, which were made in the mesio-distal direction throu-

gh the experimental roots along their long axis. The sections were stained with hematoxylin and eosin (HE) and with Masson's trichrome. The tissues were examined microscopically using a light microscope.

The inflammatory characteristics of the periodontal tissue were histologically analyzed by the presence or absence of inflammatory cells inside the subepithelial connective tissue, and by the presence or absence of epithelial projections into the connective tissue. Another aspect analyzed after the treatment, was the healing of the pocket by long junctional epithelium and its location on the root, or by connective tissue attachment. The presence or absence of cement and neoformed bone was also analyzed as well as the presence of root resorption or ankylosis. All of these evaluations were realized near the notch made in the dentin root by an endodontic file in the most apical position in the pocket.

Masson's trichrome staining was performed to identify the connective fibers inside the connective tissue and also to evaluate their directions near the root surface.

Histomorphometric analysis

This analysis was realized with a grid to determine the percentage of periodontal tissue that had filled the notch, as well as the percentage of each type found. A 400 intersection grid was aligned with its vertical axis parallel to the root surface and positioned at the bottom of the notch at 200x magnification, so that the whole notch was included inside the grid. The horizontal axis was positioned bisecting the notch. The number of grid point intersections in the defined area, which were aligned over the total tissue area, the epithelial area, and the inflamed connective tissue area or sound connective tissue area were counted. The connective tissue area exhibiting inflammation was defined as that portion of connective tissue with high rounded cell density, strongly stained in blue, with condensed nuclei, minimal cytoplasm and with a large number of blood vessels. The intersections on the connective tissue-epithelium interface were counted as connective tissue. The intersections that were aligned over empty tissue spaces, blood vessels, or artefacts were not counted.

Statistical analysis

Data obtained from periodontal repair were analyzed separately for each type of tissue: epithe-

lium, inflamed connective tissue, healthy connective tissue, and total field. The descriptive statistics from the data were presented as means and standard deviations. In this study, the dependent variable was the periodontal repair, while the independent variables were (i) treatment (TTC, CA, NaCl), (ii) sacrifice period (3, 7 and 21 days), as well as the (iii) interaction between these factors. Statistically significant differences among the means of the groups were tested using two-way analysis of variance (ANOVA) and the Tukey multiple comparisons test. Differences were regarded as statistically significant at $p < 0.05$ and the data were analyzed using a software program STATISTIX for Windows (2000, 7.0 version, Analytical Software).

RESULTS

Histological findings

The histological characteristics observed in the analyzed specimens were equivalent inside each period of sacrifice in the three groups. The gingival tissue partially filled the notch, and was covered by junctional epithelium (Figure 1). The sections of animals sacrificed three days after the treatment showed proliferation of the basal stratum of the epithelium into the areas of inflamed connective tissue and more projections towards the connective tissue (Figure 2). The connective tissue adjacent to the junctional epithelium was rich in cells, especially in its coronary portion, exhibiting some engorged blood vessels and mononuclear inflammatory cells. More apically, at the center of the gingival papilla, the connective tissue showed fewer cells and more collagen fibers with no definite arrangement and, in the region apical to the notch transseptal fibers arranged parallel, and perpendicular or oblique to root cement were observed. These characteristics were more evident in the animals sacrificed on the third day (Figure 2). However, a decrease in thickness and in the quantity of epithelial projections in the sections of animals sacrificed on the seventh and 21st days was observed, especially closer to the notch (Figures 3, 4). In animals sacrificed on the seventh and 21st days, the number of inflammatory cells was reduced and the connective tissue had become more fibrous (Figures 3, 4). None of the specimens showed new attachments, cement or bone neoformation.

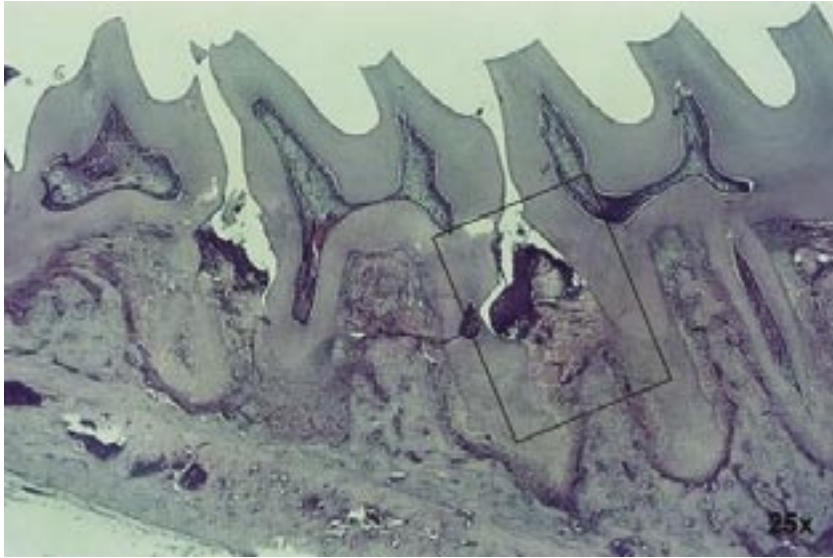


FIGURE 1– Gingival tissue partially filling the notch on the mesial of the maxillary second molar of an animal treated by scaling + irrigation with 0.9% NaCl, sacrificed three days after treatment (HE stain, original magnification 25x)

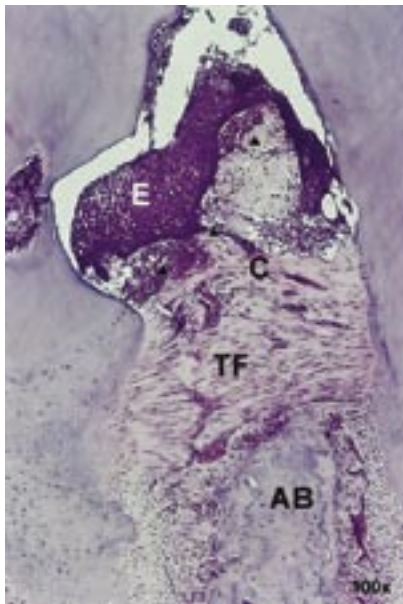


FIGURE 2 – Higher magnification of figure 1 showing a wide epithelium (E), which had some projections. The area immediately below the epithelium (E) and above the healthy gingival connective tissue (C) was infiltrated with inflammatory cells (•); TF: transeptal fibers; AB: alveolar bone (HE stain, original magnification 100x).



FIGURE 3 – Section of an animal treated with scaling + irrigation with 10% TTC-HCl and sacrificed seven days after treatment showing a narrower epithelium (E) without projections which ended at the apical limit of the notch (á). Inflammatory cells infiltrated connective tissue (•); BF: dent-alveolar bundle fibers; AB: alveolar bone (H&E stain, original magnification 100x)

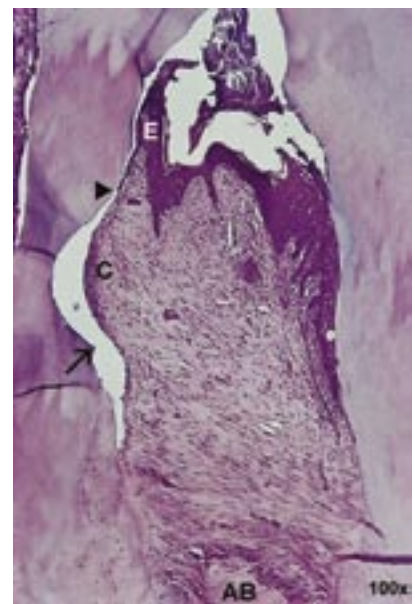


FIGURE 4– Section of an animal treated with scaling + irrigation with 0.9% NaCl and sacrificed 21 days after treatment showing junctional epithelium (E) ending at the coronal third of the notch (.,.). The connective tissue (C) was partially filling the notch until its apical limit (á); AB: alveolar bone (HE stain, original magnification 100x)

HISTOMORPHOMETRIC ANALYSIS

Total Area

The histomorphometric analysis of the notch filling was expressed as a percentage, first regarding the total area of gingival tissue. Through the ANOVA results, we were able to verify that the effect of the sacrifice period presented no statistically significant difference among the three groups ($p=0.236$) (Table 1). However, when the main effect was the solution used for irrigation, a statistically significant difference between group III and group I and, between group III and group II ($p=0.001$) was observed (Table 2).

When the interaction between the effect of sacrifice period and the irrigation solution used was analyzed for gingival filling of the notch, no statistically significant difference was observed.

Epithelium

Each individual tissue that constituted the gingival filling was also analyzed. The junctional epithelium was the first and it showed a statistically significant

difference only in regard to the sacrifice period effect ($p=0.002$) (Tables 1 and 2). The percentage of notch filling by epithelium in each period, comparing the different treatments, showed no statistically significant difference.

Inflamed connective tissue

In the evaluation of inflamed connective tissue, the results showed statistically significant difference only when the main effect was the irrigation solution ($p=0.006$) (Table 2). When the sacrifice period and its interaction with the irrigating solution were analyzed, no statistically significant difference was observed.

Healthy connective tissue

The results observed for the notch filling with connective tissue without inflammation were statistically different only for the sacrifice period effect ($p=0.007$). This difference was between three and 21 days and, between seven and 21 days (Table 1). The irrigation solution and the interaction between irrigation solution and sacrifice period showed no statistically significant difference (Table 2).

Table 1 – Data of gingival filling (%) of the notch, for animal sacrifice period and the tissue repair

Sacrifice periods (days)	Total Area	Epithelium*	Connective with inflammation	Connective without inflammation*
3	48.20±16.13	23.52±7.21 a	15.08±8.25	9.60±11.89 a
7	45.84±18.62	15.98± 7.77 b	17.80±10.58	12.06±11.40 a
21	54.84±15.96	11.58± 6.91 b	16.95±9.31	26.31± 16.27 b

* Means followed by the same letter in the column are not statistically different by the Tukey test, $p<0.05$

Table 2 – Data of gingival filling (%) of the notch, for irrigation solution and the tissue repair

Irrigation solution (ml)	Total Area*	Epithelium	Connective with inflammation*	Connective without inflammation
TCC (I)	42.69±17.43 a	17.46±7.44	11.80±6.67 a	13.43±13.99
Citric acid (II)	42.84±11.47 a	13.89±5.26	14.65±6.87 a	14.29±12.38
NaCl (III)	63.36±12.85 b	19.73±11.73	23.37±9.99 b	20.26±15.57

* Means followed by the same letter in the column are not statistically different by the Tukey test, $p<0.05$

DISCUSSION

The results observed in this study showed that the root surface conditioning associated with scaling and root planning did not improve tissue repair after periodontal therapy. The analysis of the periodontal tissue after treatment in each group was used as a reference in this study. So, as in previous studies⁶, the use of a notch to limit a specific field of observation of the histological events in the periodontal tissue repair can establish a comparative standard for the results obtained in test and control groups. The histomorphometric findings obtained with the use of a grid point revealed perceptual data of the notch filling and allowed the statistical analysis to support the descriptive histology findings¹². Histomorphometric studies have also been used to justify clinical and histological aspects observed in inflamed human gingival tissue⁹.

In descriptive histology, standards were established in order to characterize each tissue involved in the healing process. In this study, the junctional epithelium, the direction of periodontal fiber bundles and the degree of inflammation were analyzed. Our results showed that, although the total gingival tissue which filled the notch presented no statistically significant difference when the main effect was the period of sacrifice, it was possible to see changes in the ratio of each tissue inside the notch. These changes were associated with the resolution of the inflammation process, granulation tissue formation and tissue repair.

One of the aspects analyzed in the tissue change was the area of the junctional epithelium. Our study showed a reduction in the epithelial area both in the number of basal layer projections and in their thickness. However, both in the experimental groups which associated root conditioning to scaling, and in the control group, the histological sections sometimes showed that junctional epithelium followed the notch extension until the apical limit, and in others it was extended apically to the limit, over the root cement. Although Blomlöf's studies^{2,3} showed good results for root surface colonization by periodontal ligament cells, after conditioning with citric acid or EDTA, Cafesse^{5,6}, showed that conditioning with citric acid showed no statistically significant difference in connective tissue

regeneration and in bone formation. In our study none of the specimens showed cement, ligament or bone neoformation. The difficulty of performing a correct instrumentation and removing calculus and damaged cement and dentin properly may have interfered in these results. According to the Hanequin & Douillard study¹¹, the predictability of tissue response near a surface treated with citric acid is variable. This variability may be due to the fact that the removal of root tissue is not homogenous during instrumentation and the collagen matrix exposure occurs according to the depth of root tissue.

The use of tetracycline in root surface conditioning aims to act directly on the host response through its anti-inflammatory properties and indirectly through its antimicrobial properties^{18,26}. Although some *in vitro* studies have proven these properties^{15,17,24}, they are not so evident in *in vivo* studies¹⁶. In our study, the percentual of inflammatory connective tissue was equivalent in both test groups and it was lower than in the control group. The connective tissue without inflammation showed an increase in its ratio independent of the experimental or control group, showing a higher mean value on the 21st day, as can be observed in the histological sections in all groups. These results confirm the maturation that the tissue demonstrated from the first to the last observation day. The connective tissue infiltrated by inflammatory cells showed few significant changes between the sacrifice periods. However, when the treatment was analyzed, the rats which were treated with citric acid or TTC showed a lower percentage of inflamed connective tissue when compared with the NaCl group. Selvig¹⁸ showed that conditioning with citric acid did not damage periodontal healing, but Blomlöf³ considered that the pH of the conditioning solution could be fundamental to removing the smear layer without compromising periodontal tissue vitality.

Thus, the results of this study suggest that the use of acid solutions for root conditioning, do not impair periodontal tissue healing. We can, however, conclude that new attachments were not seen in any specimens and the use of acidic solution after periodontal therapy can reduce tissue inflammation and improve the tissue repair.

RESUMO

O reparo tecidual após terapia periodontal consta da cicatrização epitelial e conjuntiva pela a biocompatibilização da superfície radicular. O objetivo deste estudo foi investigar se o uso de soluções condicionantes como o cloridrato de tetraciclina (TTC-HCl) ou o ácido cítrico podem melhorar o reparo após a terapia periodontal em ratos. A doença periodontal foi induzida pela inserção de uma ligadura nos 2º molares superiores esquerdos em 36 ratos Wistar. O sítio experimental foi tratado mecanicamente e uma marcação na porção mais apical da bolsa foi preparada com uma lima endodôntica de Hedstroen. Os sítios experimentais foram então irrigados com de solução de cloridrato de tetraciclina à 10% ou, de solução de ácido cítrico à 8% ou, de solução de NaCl à 0,9% formando 3 grupos de 12 animais. Os animais foram sacrificados 3, 7, ou 21 dias após o tratamento e as peças cirúrgicas foram processadas para a análise histomorfométrica. Os resultados mostraram maturação da cicatrização de forma normal, sem regeneração nos sítios experimentais. Utilizando-se um retículo de contagem e a análise de variância a dois critérios observou-se que o tecido que preencheu a marcação não diferiu significativamente entre os sítios irrigados com soluções condicionantes. Entretanto, o tecido que preenchia a marcação era menor nestes grupos em relação ao controle e apresentava uma área ocupada por tecido conjuntivo com inflamação mais estreita. Baseado nestes resultados pode-se concluir que a irrigação com soluções ácidas pode melhorar o reparo após a terapia periodontal.

PALAVRAS CHAVE

Tetraciclina; cavidade de polpa dentária; ácido cítrico, reparo periodontal

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Lucilene Hernandes Ricardo
Avenida Antônio Cozzi, nº 565
Residencial Lessa
phone: 55 12 243-1504 or 55 12 2422469
e-mail: lumello1@uol.com.br
12.400 – 000 – Pindamonhangaba – SP