UNIVERSIDADE ESTADUAL PAULISTA
"Júlio de Mesquita Filho"

CRISTIANE CANTIGA DA SILVA

Efeito da fibrose hepática sobre a severidade da periodontite apical: análise histológica e imunoistoquímica em ratos

ARAÇATUBA

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Dissertação apresentada à Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista "Júlio de Mesquita Filho" - UNESP como parte dos requisitos para obtenção do título de Mestre em Ciência Odontológica, área de concentração em Endodontia.

Orientador: Prof. Dr. Luciano Tavares Angelo Cintra

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Identificação

Nascida aos **15 dias de março de 1991** em Envira, AM.

Filha de **João Rates da Silva e Luzia Cantiga de Souza**.

2009 – 2013 – Curso de Graduação

Concluiu o curso de **Graduação em Odontologia**, na Universidade do Norte – UNINORTE, em dezembro de 2013.

2014 – 2016 – Curso de Especialização

Concluiu o curso de **Especialização em Endodontia** pela Universidade do Estado do Amazonas – UEA, em janeiro de 2016.

2018 – 2020 – Curso de Mestrado

Concluiu o **Curso de Mestrado em Ciência Odontológica, área de concentração Endodontia**, na Faculdade de Odontologia do Campus de Araçatuba, Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP, sob orientação do professor Luciano Tavares Angelo Cintra.

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“Os dias prósperos não vêm por acaso.

Nascem de muito trabalho e persistência.”

Henry Ford

CANTIGA-SILVA, C. **Efeito da fibrose hepática na severidade da periodontite apical: análise histológica e imunoistoquímica em ratos.** 2020. 50 f. (Dissertação). Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba, 2020.

RESUMO

A inter-relação das alterações sistêmicas com o desenvolvimento e progressão de infecções orais como a periodontite apical tem sido objeto de intensos estudos nos últimos anos. O objetivo deste trabalho foi verificar a influência da fibrose hepática (FH) na severidade da periodontite apical (PA) em ratos Wistar. Quarenta ratos foram divididos em 4 grupos (n=10): Grupo C - ratos controle; Grupo PA - ratos portadores de PA; Grupo FH - ratos portadores de FH; Grupo PA+FH - ratos portadores de PA e FH. A FH foi induzida pelos métodos químico e cirúrgico associados. Foi administrado Tetracloreto de Carbono (CCl_4) na dosagem de 0,2ml/100g de peso corporal, 2 vezes por semana, via intraperitoneal, e durante todo o experimento (60 dias). Após 30 dias do início da administração da droga, os animais foram submetidos à cirurgia de ligadura do ducto biliar. Em seguida, as a polpa dentária dos primeiros e segundos molares superiores e inferiores direito foram expostas pelo período de 30 dias para desenvolvimento da PA. Ao final do experimento os animais foram eutanaziados e as maxilas, assim como os fígados, coletados para análise em microscopia de luz. O tecido hepático foi analisado em coloração de hematoxilina e eosina (H&E) e Picrosírius red para comprovar a fibrose hepática e as maxilas processadas para análise histológica, histométrica e imunoistoquímica para IL-1β, IL-6, e TNF-α. Os resultados obtidos foram analisados e comparados por testes estatísticos específicos para cada caso com nível de significância de 5% ($p < 0.05$). A FH foi confirmada pela análise histológica dos fígados nos grupos FH e PA+FH que apresentaram hepatócitos necrosados, desorganização vascular e intensa deposição de colágeno no parênquima hepático, formando pontes de fibrose. Quanto à periodontite apical observou-se infiltrado inflamatório moderado no grupo PA e intenso no PA+FH ($p < 0.05$). A análise histométrica mostrou maiores áreas de reabsorção óssea periapical no grupo PA+FH em comparação ao grupo

PA ($p < 0.05$). A análise imunoistoquímica revelou maior imunomarcação para citocinas IL-1β, IL-6 e TNF-α no grupo PA+FH quando comparado ao grupo PA ($p < 0.05$). Conclui-se que a FH influencia na severidade da periodontite apical, exacerbando o infiltrado inflamatório, por meio do aumento das citocinas IL-1β, IL-6 e TNF-α, e aumentando a reabsorção óssea periapical.

Palavras-chave: Periodontite apical. Fibrose hepática. Inflamação. Citocinas pró inflamatórias.

CANTIGA-SILVA, C. **Effect of liver fibrosis on the severity of apical periodontitis: histological and immunohistochemical analysis in rats.** 2020. 50 f. (Dissertação). Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba, 2020.

ABSTRACT

The interrelationship between systemic disorders and oral infections such as apical periodontitis has been the subject of intense studies in recent years. The aim of this study was to evaluate the influence of liver fibrosis (LF) on the severity of apical periodontitis (AP) in Wistar rats. Forty rats were divided into 4 groups (n=10): Group C - control rats; AP group - rats with AP; LF Group - rats with LF; AP+LF Group - rats with AP and LF. LF was induced by the association of chemical and surgical methods. Carbon tetrachloride (CCl_4) was administered at a dosage of 0.2ml/100g of body weight, twice a week, intraperitoneally, and throughout the experiment (60 days). After 30 days from the beginning of the drug administration, the animals were submitted to bile duct ligation surgery and the dental pulps of the first and second right maxillary and mandibular molars were exposed to induce AP in a period of 30 days. At the end of the experiment, the animals were killed and the jaws, as well as the livers, were collected for analysis under light microscopy. The liver tissue was analyzed in Hematoxilin and Eosin (H&E) and Picrosírius red staining to confirm the liver fibrosis. The jaws were processed for histological, histometric and immunohistochemical analysis for IL-1β, IL-6, and TNF-α. The results obtained were analyzed and compared by specific statistical tests ($p < 0.05$). LF was confirmed by histological analysis of the liver in the LF and AP+LF groups, which presented necrotic hepatocytes, vascular disorganization and intense collagen deposition in the liver parenchyma, forming fibrosis bridges. A moderate inflammatory infiltrate was observed, in the AP group and intense in the AP+LF ($p < 0.05$). Histometric analysis showed greater areas of periapical bone resorption in the AP+LF group compared to the AP group ($p < 0.05$). The immunohistochemical analysis revealed greater immunolabeling for cytokines IL-1β, IL-6 and TNF-α in the AP+LF group when

compared to the AP group ($p < 0.05$). It is concluded that LF influences on the severity of apical periodontitis, exacerbating the inflammatory infiltrate, by increasing the cytokines IL-1β, IL-6 and TNF-α, and intensifying the periapical bone resorption.

Keywords: Apical periodontitis. Liver fibrosis. Inflammation. Pro inflammatory cytokines.

S U M Á R I O

1. ARTIGO - THE INFLAMMATORY PROFILE OF APICAL PERIODONTITIS ASSOCIATED WITH LIVER FIBROSIS: HISTOLOGICAL AND IMMUNOHISTOCHEMICAL ANALYSIS. 1

ANEXOS

1. Artigo

The inflammatory profile of apical periodontitis associated with liver fibrosis: histological and immunohistochemical analysis

Journal of Endodontics

The inflammatory profile of apical periodontitis associated with liver fibrosis: histological and immunohistochemical analysis

ABSTRACT

Introduction: The study evaluated the effects of liver fibrosis (LF) in the modulation of pro inflammatory mediators in apical periodontitis (AP) and periapical bone resorption. **Methods:** Forty male Wistar rats were distributed into four groups: C - control, AP - rats with AP, LF - rats with LF, AP+LF - rats with AP and LF. LF was induced by the administration of carbon tetrachloride for eight weeks associated with surgery procedure for bile duct ligation for four weeks; AP was induced by dental pulp exposure to the oral environment for 30 days. In the euthanasia, jaws and liver were removed. The livers were analyzed in Hematoxilin and Eosin (H&E) and Picrosirius red staining to confirm fibrosis. The jaws were analyzed in H&E staining and immunohistochemical assays for interleukin (IL) -1β, IL-6 and tumor necrosis factor alpha (TNF-α). The Student *t* test and Mann-Whitney *U* test and were used for statistical analysis ($P < .05$). **Results**: The inflammatory infiltrate was moderate in AP and severe in AP+LF ($P < .05$). The periapical bone resorption was larger in AP+LF than AP group ($P < .05$). IL-1β, IL-6 and TNF-α levels were higher in the AP+LF groups when compared to the AP group ($P < .05$). **Conclusion:** Liver fibrosis modulates apical periodontitis increasing the inflammatory infiltrate by changing the pro inflammatory cytokines immunolabeling as well as enhanced the periapical bone resorption.

KEY WORDS

Apical periodontitis; liver fibrosis; inflammation; cytokines

INTRODUCTION

Apical periodontitis (AP) is a common pathology that affects the periapical tissues. It is caused by a persistent inflammatory reaction in response to the action of infectious agents present in the root canal (1). The pathogenesis of AP depends on the control of the host's immune response to microorganisms; Consequently to intensity of the inflammatory infiltrate and the the release of pro and anti-inflammatory cytokines (2).

The AP is characterized by periapical bone resorption that happens through the interaction of inflammatory stimuli that activates the cells responsible for bone resorption (3). In addition, the immune response may be exacerbated by systemic complications such as diabetes, aggravating the development of AP (4). Recently, a study revealed the presence of periapical radioluscence in more than half of patients with liver cirrhosis, showing that liver changes can influence in AP (5). In this context, a previous study has shown that more than one focus of AP in humans is common (6). Furthermore, animal model studies revealed that the presence of multiple AP foci can influence on systemic disorders (7, 8).

Liver diseases affect thousands of people around the world by raising mortality rate in developed countries. The World Health Organization affirms that Viral Hepatitis C affects more than 70 million people worldwide, followed by non-alcoholic fatty liver disease (9). These chronic diseases lead the liver to steatosis, fibrosis, and cirrhosis. The liver fibrosis (LF) is modulated by oxidative stress, increased tumor growth factor-β1 (TGF-β1) (10) and the activation of kupffer cells that release inflammatory mediators such as interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNF-α) (11) which will progress to cirrhosis if not treated (10).

Pro inflammatory cytokines are secreted by different type of cells, just like macrophages, odontoblasts and osteoclasts (12); Also, they play a crucial role in the development and persistence of AP (13). Elevated levels of IL-1β were related to higher levels of periapical bone resorption in systemic complications (14), as well as the production of other pro inflammatory mediators like the IL-6 and TNF-α (15). In the liver, these cytokines act as mediators in the transition from the beginning of liver injury to fibrogenesis and consequently to fibrosis (10); Moreover, the IL-1β could be detected in high serum levels in patients with chronic liver diseases, proving its relation with the evolution of hepatic conditions (16).

Considering that the IL-1β, IL-6, and TNF-α are related to the development of liver fibrosis (10, 11, 16), as well as to the development of apical periodontitis (4), this study proposed the evaluation of the effects of LF in severity of the apical periodontitis. The null hypothesis is that LF do not influence on pro inflammatory mediators immunolabeling and periapical bone resorption of AP.

MATERIALS AND METHODS

Experimental design

Forty male rats (*Rattus norvegicus albinus,* Wistar), with one month old, with an average of 100 g of weight were selected for this study (n = 10). The animals were housed in temperature-controlled rooms and received water and food ad libitum. The experimental procedures were approved and conducted in accordance to the Animal Ethics Committee, Universidade Estadual Paulista 00430-2018 and in compliance with the U.K. Animals (Scientific Procedures) Act 1986.

The following groups were assigned: C - control, AP - rats with AP, LF - rats with LF, AP+LF - rats with AP and LF.

Induction of liver fibrosis

Twenty animals received 40% carbon tetrachloride (CCl_4) (SIGMA-ALDRICH St. Louis - Missouri, USA) diluted in olive oil (SIGMA-ALDRICH St. Louis, Missouri, USA), 0.2 ml / 100 g twice weekly (17), by intraperitoneal injection during 60 days (18) (day 0). Thirty days after the first administration of CCl_4 (day 30), the animals were anesthetized with intramuscular administration of ketamine, 87 mg / kg (Francotar - Virbac do Brasil Ind. E Com. Ltda, Roseira, Brazil) an xylazine, 13 mg / kg (Rompum - Bayer SA, Sao Paulo, Brazil) and then, they were submitted to the bile duct ligature surgery procedure (BDL). The animals were kept alive for 30 days after the surgical induction liver fibrosis (19).

Induction of apical periodontitis

The pulp exposure for induction of periapical lesions was performed minutes before BDL surgery (day 30). The dental pulps of the first and second right maxillary and mandibular molars of 20 rats were exposed on the mesial surface by means of a carbon steel drill - Ln Long Neck (Dentsply/Maillefer, Ballaigues, Switzerland); The pulps got exposed for 30 days, allowing the periapical lesion formation (20).

Sample obtaining and processing

At the end of the experiment (day 60), the animals were 3 months old, with an average weight of 400 g. After euthanasia, the right lobe of the livers (21) and jaws containing maxillary molars were collected and fixed in a buffered solution with 4% formalin, at neutral pH, during the first 22 hours. The specimens were washed in running water for a period of 12 hours. The livers were subjected to conventional histological processing to obtain tissue sections embedded in paraffin. After being

washed, the jaws were decalcified in EDTA 10% (SIGMA-ALDRICH St. Louis - Missouri, USA).

After inclusion, semi-serial tissue sections of 4-µm thickness from livers were prepared and stained with Hematoxylin and Eosin (H&E) and Picrosirius Red (PSR). The first molar was sectioned semi serially (thickness = 4 µm) along its longitudinal axis (22). The Sections were stained with H&E techniques and submitted to immunohistochemistry using the indirect immunoperoxidase technique with the following primary antibodies: rabbit anti-IL-1β (10 *µ*g/mL ab9722, Abcam, Cambridge, MA) rabbit anti-IL-6 (1:200 SC 1265, Santa Cruz Biotechnology, Santa Cruz, CA) and goat anti-TNF-a (1:100 SC 1348, Santa Cruz Biotechnology, Santa Cruz, CA) The immunohistochemical processing followed the protocol described by Cintra et al 2016 (20).

Histological, Histometric and Immunohistochemical Analysis

The liver analysis was performed to confirm hepatic lesions and to characterize the type of alteration observed: a) lobular inflammation; b) hepatocellular necrosis; c) inflammation of the portal tract; d) pericellular fibrosis, portal fibrosis and fibrosis bridge (11).

The intensity of periapical inflammation was analyzed in H&E staining by assigning scores graded as follows: no inflammation (score 1: 0 or few inflammatory cells), mild inflammation (score 2: <25 inflammatory cells), moderate inflammation (score 3: 25–125 inflammatory cells), and severe inflammation (score 4: >125 inflammatory cells) (20).

The histometric analysis was performed in the AP and AP+LF groups to verify if there was a difference in periapical bone loss of AP rats associated with the

presence of LF. The Leica LAS X (Leica Microsystems, Nussloch - Germany) was used and the values were expressed in square micrometers (μm^2) obtained in area measurements (22).

For immunohistochemical analyses, three histologic sections of the jaws were used for each animal, and positive immunoreactivity was defined as a brownish color in the cytoplasm and extracellular matrix. For enumeration of the immunoreactive cells, a standardized guide frame was overlaid on the captured images. The intensity was obtained by the observation of immunoreactive cells and extracellular matrix labeling; It was performed semiquantitative analysis applied on scores according to the immunolabeling pattern of IL-1β, IL-6 and TNF-α. The scores were adapted from Cosme-Silva et al 2019 (23). Score 0: no immunolabeling (total absence of immunoreactive); Score 1: <20% - immunoreactive cells and low extracellular matrix labeling; Score 2: >20% and <40% - immunoreactive cells and low extracellular matrix labeling; Score 3: >40% and <60% - immunoreactive cells and moderate extracellular matrix labeling; Score 4: >60% and <80% - immunoreactive cells and high extracellular matrix labeling; Score 5: >80% - immunoreactive cells and high extracellular matrix labeling.

Statistical analysis

The total values were tabulated for each experimental group and the data were analyzed by a single calibrated operator in a blinded manner. For analysis of parametric data regarding to the area of periapical lesion, the Student *t* test was used. Nonparametric data were analyzed by performing multiple comparisons with the Mann-Whitney *U* test applied at a significance level of 5% ($P < .05$). The Power test was 92.9%.

RESULTS

Analysis of Liver Fibrosis

The induction of liver fibrosis was confirmed in LF and AP+LF groups. In C and AP groups, the analysis revealed aspects of normality in the parenchyma characterized by the absence of cell death, vascular organization, as well as absence of collagen. The livers of the LF and AP+LF animals presented lobular Inflammation, hepatocellular necrosis, alteration of the vascular architecture with the presence of numerous blood vessels and portal tract inflammation, and collagen deposited in pericellular space forming portal bridges, revealing the presence of hepatic fibrosis (Fig. 1).

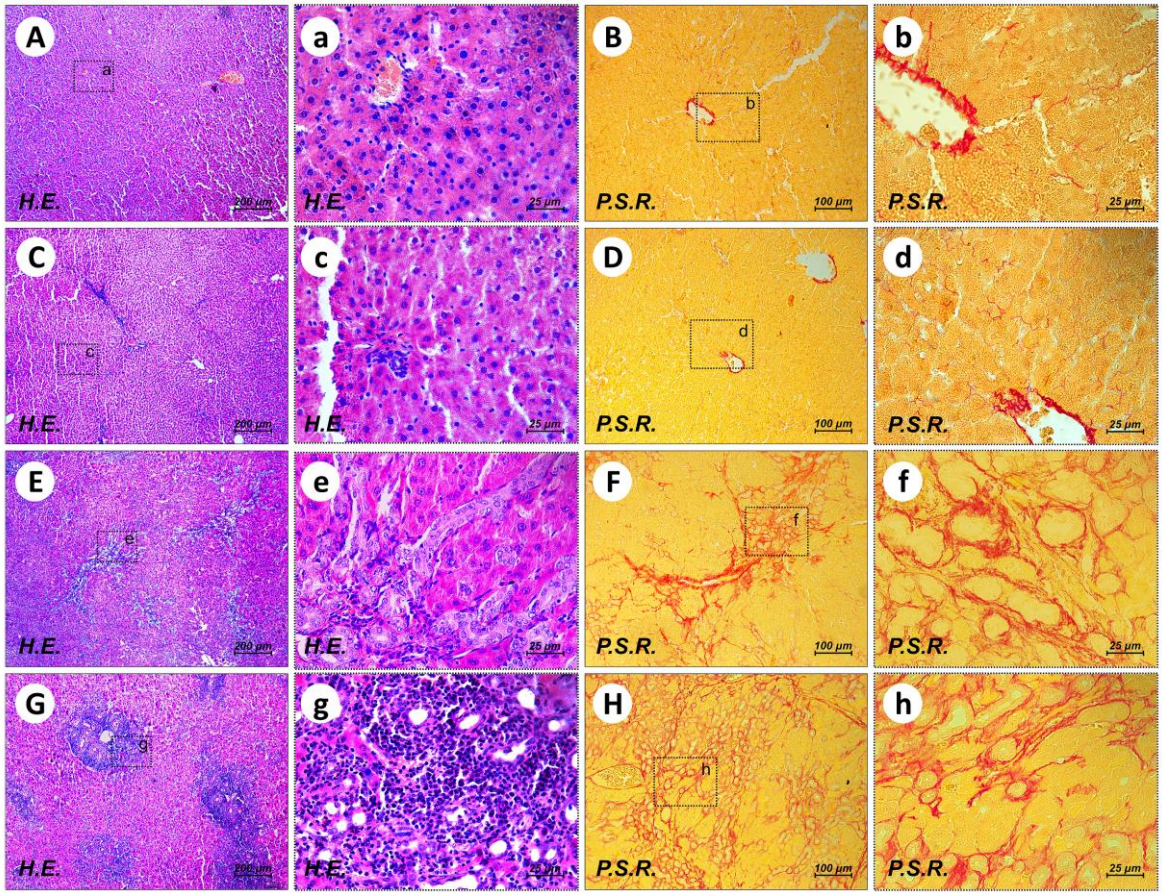

FIGURE 1. Representative images of the right lobe of the liver after 60 days of experiment stained with H&E and PSR. H&E staining in C (A, a) and AP (C, c) groups show cellular and structural organization in the parenchyma, absence of necrotic cells, unchanged

vascular plexus and absence of collagen deposition between hepatocytes. LF (E, e) and AP+LF (G, g) group reveal cellular necrosis, kupffer cells throughout the parenchyma, disruption of blood vessels as well as intense bridging collagen deposition characterizing liver fibrosis. PSR staining in C (B, b) and AP (D, d) group show that collagen is present only on the blood vessel wall, revealing normal structure of the parenchyma. The LF (F, f) and AP+LF (H, h) group have large collagen deposition forming fibrosis bridges and septa in the liver parenchyma. (H&E and PSR staining, A - H 100x; a - h 400x increase).

Histological, Histometric and Immunohistochemical Analysis of Apical Periodontitis

AP induction was confirmed by histologic examination of the jaws in the AP and AP+LF groups. No signs of pulpal and periapical inflammation were observed in C and LF groups. In AP and AP+LF, the dental pulp showed signs of total necrosis after 30 days of pulp exposure, and the presence of apical periodontitis in these group was also observed. In addition, the inflammatory infiltrate of the lesions consisted of neutrophils and mononuclear cells. The intensity of the inflammatory infiltrate was higher in the AP+LF group (score 4), compared to the AP (score 3) ($P < .05$) (Fig. 2; Table 1). The histometric analysis showed that the AP+LF had higher bone resorption when compared to the AP group ($P < .05$) (Fig 2; Table 1).

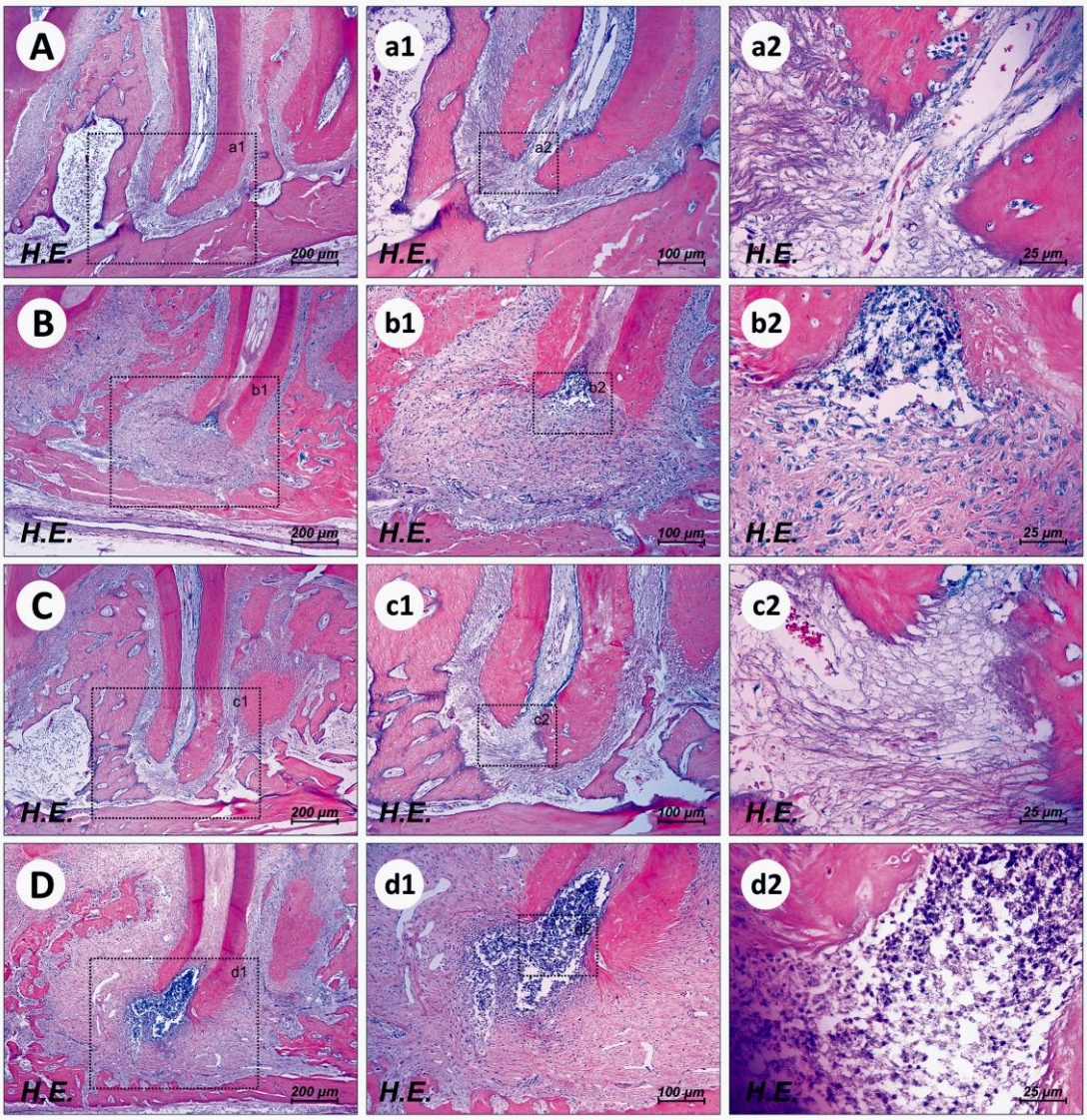

FIGURE 2. Representative images of histological analysis of the periapical region after 30 days of pulp exposure. H&E staining in C group (A, a1, a2) showed the periapical region free of inflammatory infiltrate; AP group (B, b1, b2) with a moderate inflammatory infiltrate around the apex, disruption of the periodontal ligament and bone resorption area; LF group (C, c1, c2) denoting similarly to C group, periapical region free of inflammatory infiltrate; AP+LF group (D, d1, d2) presenting intense inflammatory infiltrate, as well as extensive periapical bone resorption area. (A - D, 50x; a1 – d1, 100x; a2 – d2, 400x increase).

Immunohistochemical analysis revealed increased levels of IL-1β, IL-6 and TNF-α cytokines in the AP and AP+LF groups with a significant increase in

immunostaining in the AP+LF group revealing high or extremely high scores when compared to the AP group ($P < .05$) (Fig. 3; Table 1).

Table 1. The Median Scores, Interquartile Ranges, Mean, Standard Deviation (SD), and *P* Values of Histologic, Immunohistochemical and Histometric findings in Rats from both groups.

Histologic criteria	**Experimental groups**				**Statistical analysis**
	C	**AP**	**LF**	**AP+LF**	
Inflammatory infiltrate	1 (1-1)	3 (2-4)[a]	1 (1-1)	4 (3-4)[b]	**Mann-Whitney *U* test** *P* = .021
Immunoreactive cells					
IL-1β	1 (1-1)	2 (2-3)[a]	1 (1-1)	4 (3-4)[b]	*P* = .001
IL-6	1 (1-2)	3 (3-4)[a]	2 (1-2)	5 (4-5)[b]	*P* = .011
TNF-α	1 (1-1)	2 (2-3)[a]	1 (1-1)	4 (3-5)[b]	*P* = .007
Histometric analysis (mean ± SD)	13.20 ± 1.67	87.40 ± 12,77[a]	12.78 ± 1.71	120,87 ± 25.96[b]	**Student *t* test** *P* = .002

C, control; AP, apical periodontitis; LF, liver fibrosis; IL, interleukin; TNF, tumor necrosis factor.
Different superscript letters represent statistically significant differences (AP vs AP+LF), $P < .05$

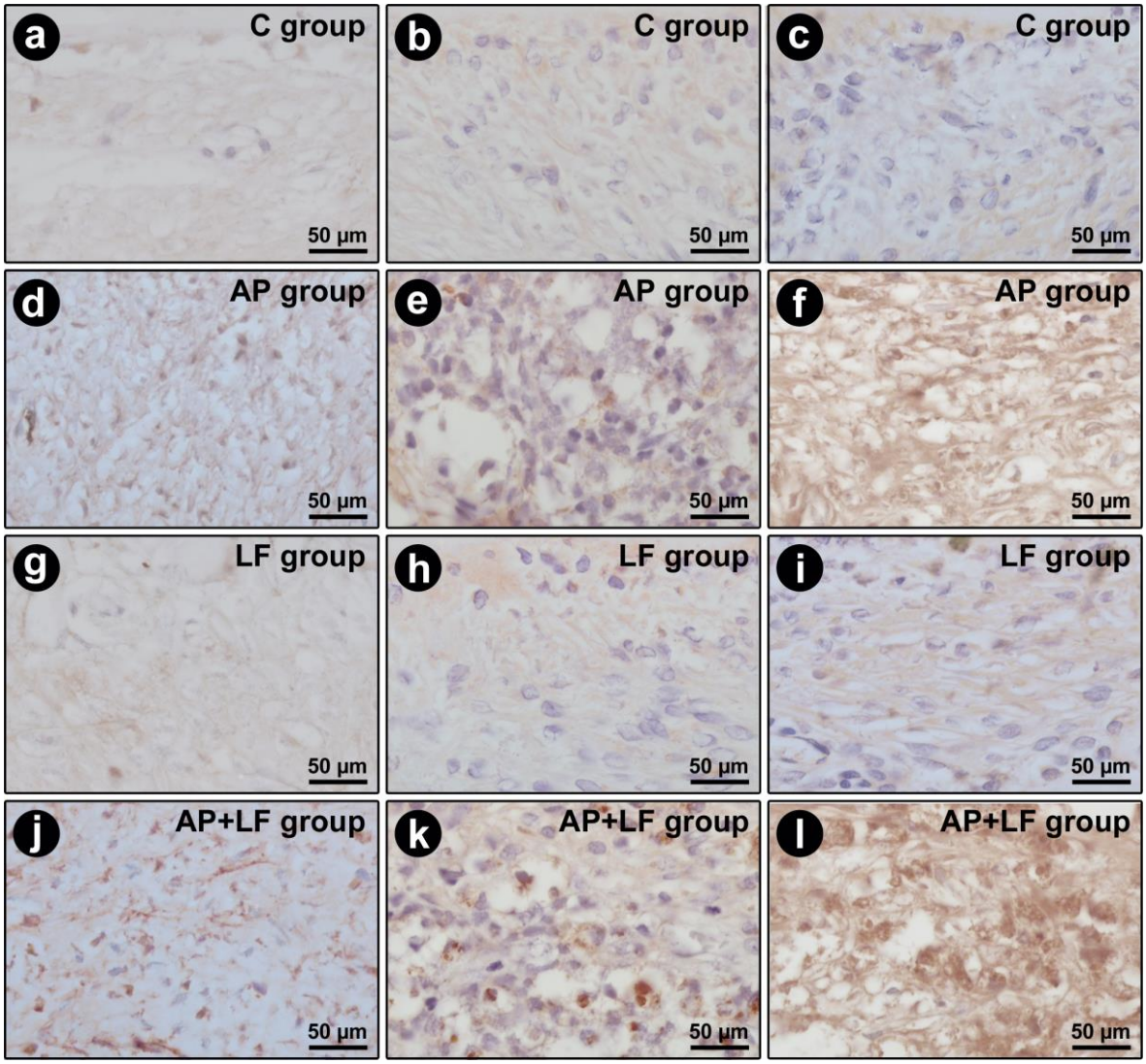

FIGURE 3. Representative images of immunohistochemical analysis of the periapical region 30 days after pulp exposure. (a - l) immunohistochemistry images of C, AP, LF and AP+LF groups. Immunolabeling pattern for cytokines IL-1β (a, d, g, j); IL-6 (b, e, h, k) and TNF-α (c, f, I, l). (H&E staining, 1000x increase).

DISCUSSION

The influence of LF on AP examining the inflammatory profile, periapical bone resorption and pro inflammatory cytokine showed that hepatic disease altered the intensity of the inflammatory infiltrate, promoted the increase of the periapical bone resorption and cytokines levels of IL-1β, IL-6 and TNF-α on peripical lesions. Thus, the null hypothesis was rejected.

Different methods of liver fibrosis induction in rats have been studied for decades; The administration of CCl_4 (17, 24) and BDL surgery are among the classical methods (19, 25). The damage to hepatocytes causes the release of free radicals that activate kupffer cells to produce pro and anti-inflammatory cytokines that control the development and progression of liver inflammation and other lesions (26). In cases where the lesion is sustained, chronic inflammation and extracellular matrix accumulation lead to a progressive replacement of the normal liver parenchyma with scar tissue (27). In our research, the administration of CCl_4 in low concentration and lower volume associated with BDL, allowed the research to be safely conducted, installing an advanced liver fibrosis associated or not with the AP without animals loss during the experimental period.

The method of AP induction was based on previous studies (28, 29). In this assay it was previously used a standardized method to analyze the interrelation of apical periodontitis with systemic diseases (4, 20, 22, 30). The confirmation of apical periodontitis by histological analysis showed areas of pulp necrosis, inflammatory infiltrate and periapical bone resorption in the AP and AP + LF groups.

In our results, the immunolabeling of IL-1β, IL-6 and TNF-α was higher in animals with fibrosis associated with apical periodontitis. IL-1β stimulates lymphocytes, enhances neutrophils and strengthen leukocyte adhesion (1) acting as a key regulator of host response to microbial infection playing a key role in controlling resorption and periapical bone formation (14). In periapical lesions there is a considerable increase of TNF-α in the tissues surrounding the dental element, and it can be assumed that this cytokine may induce local osteoclastic activity (31). The IL-6 influences inflammatory responses by stimulating osteoclast formation contributing to systemic increase and bone resorption (20). This cytokine is

produced in bone by the stimulation of IL-1β and TNF-α (32), which reaffirms our findings that show the increase in IL-6 levels in the AP+LF group associated with exacerbation of IL-1β and TNF-α in the periapical bone resorption.

We observed that liver fibrosis play important role in the immunolabeling of IL-1β, IL-6 and TNF-α inducing the increase of inflammatory infiltrate as well as periapical bone resorption of AP as observed in other studies that evaluated systemic diseases associated with apical periodontitis (4, 28, 30). Moreover, these cytokines were reported to the hepatic inflammatory response, and play an important role in the development and progression of liver fibrosis (10), influencing in the activation of hepatic stellar cells to produce scar-forming collagen (33), besides being related to periapical bone resorption (13).

Some studies proved that systemic conditions such as diabetes, renal disfunction and cirrhosis can influence in the endodontic inflammatory response (5, 6, 34) and this study was able to confirm that LF is also a systemic disease that is responsible for worsening apical periodontitis. Since these diseases have the ability of influencing in the severity of AP, further studies are needed to elucidate how these alterations can interfere in the success of endodontic therapy.

CONCLUSION

Liver fibrosis potentialized the severity of apical periodontitis increasing the inflammatory infiltrate by increasing the levels of pro inflammatory cytokines IL-1β, IL-6 and TNF-α as well as enhanced the periapical bone resorption.

ACKNOWLEDGMENTS

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The authors deny any conflicts of interest related to this study.

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Anexos

Guia para autores – Journal of Endodontics

General Points on Composition

1. Authors are strongly encouraged to analyze their final draft with both software (e.g., spelling and grammar programs) and colleagues who have expertise in English grammar. References listed at the end of this section provide a more extensive review of rules of English grammar and guidelines for writing a scientific article. Always remember that clarity is the most important feature of scientific writing. Scientific articles must be clear and precise in their content and concise in their delivery since their purpose is to inform the reader. The Editor reserves the right to edit all manuscripts or to reject those manuscripts that lack clarity or precision, or have unacceptable grammar or syntax. The following list represents common errors in manuscripts submitted to the *JOE*:

2. The paragraph is the ideal unit of organization. Paragraphs typically start with an introductory sentence that is followed by sentences that describe additional detail or examples. The last sentence of the paragraph provides conclusions and forms a transition to the next paragraph. Common problems include one-sentence paragraphs, sentences that do not develop the theme of the paragraph (see also section “c” below), or sentences with little to no transition within a paragraph.

3. Keep to the point. The subject of the sentence should support the subject of the paragraph. For example, the introduction of authors’ names in a sentence changes the subject and lengthens the text. In a paragraph on sodium hypochlorite, the sentence, “In 1983, Langeland et al., reported that sodium hypochlorite acts as a lubricating factor during instrumentation and helps to flush debris from the root canals” can be edited to: “Sodium hypochlorite acts as a lubricant during instrumentation and as a vehicle for flushing the generated debris (Langeland et al., 1983)." In this example, the paragraph’s subject is sodium hypochlorite and sentences should focus on this subject.

4. Sentences are stronger when written in the active voice, *i.e.*, the subject performs the action. Passive sentences are identified by the use of passive verbs such as “was,” “were,” “could,” etc. For example: “Dexamethasone was found in this study to be a factor that was associated with reduced inflammation,” can be edited to: “Our results demonstrated that dexamethasone reduced inflammation.”

Sentences written in a direct and active voice are generally more powerful and shorter than sentences written in the passive voice.

5. Reduce verbiage. Short sentences are easier to understand. The inclusion of unnecessary words is often associated with the use of a passive voice, a lack of focus or run-on sentences. This is not to imply that all sentences need be short or even the same length. Indeed, variation in sentence structure and length often helps to maintain reader interest. However, make all words count. A more formal way of stating this point is that the use of subordinate clauses adds variety and information when constructing a paragraph. (This section was written deliberately with sentences of varying length to illustrate this point.)

6. Use parallel construction to express related ideas. For example, the sentence, “Formerly, endodontics was taught by hand instrumentation, while now rotary instrumentation is the common method,” can be edited to “Formerly, endodontics was taught using hand instrumentation; now it is commonly taught using rotary instrumentation.” The use of parallel construction in sentences simply means that similar ideas are expressed in similar ways, and this helps the reader recognize that the ideas are related.

7. Keep modifying phrases close to the word that they modify. This is a common problem in complex sentences that may confuse the reader. For example, the statement, “Accordingly, when conclusions are drawn from the results of this study, caution must be used,” can be edited to “Caution must be used when conclusions are drawn from the results of this study.”

8. To summarize these points, effective sentences are clear and precise, and often are short, simple and focused on one key point that supports the paragraph’s theme.

9. Authors should be aware that the *JOE* uses iThenticate, plagiarism detection software, to assure originality and integrity of material published in the *Journal*. The use of copied sentences, even when present within quotation marks, is highly discouraged. Instead, the information of the original research should be expressed by new manuscript author’s own words, and a proper citation given at the end of the sentence. Plagiarism will not be tolerated and manuscripts will be rejected, or papers withdrawn after publication based on unethical actions by the authors. In addition, authors may be sanctioned for future publication.

Organization of Original Research Manuscripts

Please Note: *All abstracts should be organized into sections that start with a one-word title (in bold),* i.e.*, Introduction, Methods, Results, Conclusions, etc., and should not exceed more than 250 words in length.*

1. **Title Page:** The title should describe the major emphasis of the paper. It should be as short as possible without loss of clarity. Remember that the title is your advertising billboard—it represents your major opportunity to solicit readers to spend the time to read your paper. It is best not to use abbreviations in the title since this may lead to imprecise coding by electronic citation programs such as PubMed (*e.g.*, use "sodium hypochlorite" rather than NaOCl). The author list must conform to published standards on authorship (see authorship criteria in the Uniform Requirements for Manuscripts Submitted to Biomedical Journals at icmje.org). The manuscript title, name and address (including email) of one author designated as the corresponding author. This author will be responsible for editing proofs and order reprints when applicable. The contribution of each author should also be highlighted in the cover letter.

2. **Abstract:** The abstract should concisely describe the purpose of the study, the hypothesis, methods, major findings, and conclusions. The abstract should describe the new contributions made by this study. The word limitations (250 words) and the wide distribution of the abstract (*e.g.*, PubMed) make this section challenging to write clearly. This section often is written last by many authors since they can draw on the rest of the manuscript. Write the abstract in past tense since the study has been completed. Three to ten keywords should be listed below the abstract.

3. **Introduction:** The introduction should briefly review the pertinent literature in order to identify the gap in knowledge that the study is intended to address and the limitations of previous studies in the area. The purpose of the study, the tested hypothesis and its scope should be clearly described. Authors should realize that this section of the paper is their primary opportunity to establish communication with the diverse readership of the *JOE*. Readers who are not expert in the topic of the manuscript are likely to skip the paper if the introduction fails to succinctly summarize the gap in knowledge that the study addresses. It is important to note

that many successful manuscripts require no more than a few paragraphs to accomplish these goals. Therefore, authors should refrain from performing the extensive review or the literature, and discuss the results of the study in this section.

4. **Materials and Methods:** The objective of the materials and methods section is to permit other investigators to repeat your experiments. The four components of this section are the detailed description of the materials used and their components, the experimental design, the procedures employed, and the statistical tests used to analyze the results. The vast majority of manuscripts should cite prior studies using similar methods and succinctly describe the essential aspects used in the present study. Thus, the reader should still be able to understand the method used in the experimental approach and concentration of the main reagents (*e.g.*, antibodies, drugs, etc.) even when citing a previously published method. The inclusion of a "methods figure" will be rejected unless the procedure is novel and requires an illustration for comprehension. If the method is novel, then the authors should carefully describe the method and include validation experiments. If the study utilized a **commercial product**, the manuscript must state that they either followed manufacturer's protocol or specify any changes made to the protocol. If the study used an ***in vitro*** model to simulate a clinical outcome, the authors must describe experiments made to validate the **model**, or previous literature that proved the clinical relevance of the model. Studies on **humans** must conform to the Helsinki Declaration of 1975 and state that the institutional IRB/equivalent committee(s) approved the protocol and that informed consent was obtained after the risks and benefits of participation were described to the subjects or patients recruited. Studies involving **animals** must state that the institutional animal care and use committee approved the protocol. The statistical analysis section should describe which tests were used to analyze which dependent measures; p-values should be specified. Additional details may include randomization scheme, stratification (if any), power analysis as a basis for sample size computation, drop-outs from clinical trials, the effects of important confounding variables, and bivariate versus multivariate analysis.

5. **Results:** Only experimental results are appropriate in this section (*i.e.*, neither methods, discussion, nor conclusions should be in this section). Include only those data that are critical for the study, as defined by the aim(s). Do not include all available data without justification; any repetitive findings will be rejected from

publication. All Figures, Charts, and Tables should be described in their order of numbering with a brief description of the major findings. The author may consider the use of supplemental figures, tables or video clips that will be published online. Supplemental material is often used to provide additional information or control experiments that support the results section (*e.g.*, microarray data).

6. **Figures:** There are two general types of figures. The first type of figures includes photographs, radiographs or micrographs. Include only essential figures, and even if essential, the use of composite figures containing several panels of photographs is encouraged. For example, most photos, radio- or micrographs take up one column-width, or about 185 mm wide X 185 mm tall. If instead, you construct a two columns-width figure (*i.e.*, about 175 mm wide X 125 mm high when published in the *JOE*), you would be able to place about 12 panels of photomicrographs (or radiographs, etc.) as an array of four columns across and three rows down (with each panel about 40 X 40 mm). This will require some editing to emphasize the most important feature of each photomicrograph, but it greatly increases the total number of illustrations that you can present in your paper. Remember that each panel must be clearly identified with a letter (*e.g.*, “A,” “B,” etc.), in order for the reader to understand each individual panel. Several nice examples of composite figures are seen in recent articles by Jeger et al (J Endod 2012;38:884–888); Olivieri et al., (J Endod 2012;38:1007 1011); Tsai et al (J Endod 2012;38:965–970). Please note that color figures may be published at no cost to the authors and authors are encouraged to use color to enhance the value of the illustration. Please note that a multi-panel, composite figure only counts as one figure when considering the total number of figures in a manuscript (see section 3, below, for the maximum number of allowable figures).The second type of figures is graphs (*i.e*., line drawings including bar graphs) that plot a dependent measure (on the Y-axis) as a function of an independent measure (usually plotted on the X axis). Examples include a graph depicting pain scores over time, etc. Graphs should be used when the overall trend of the results are more important than the exact numerical values of the results. For example, a graph is a convenient way of reporting that an ibuprofen-treated group reported less pain than a placebo group over the first 24 hours, but was the same as the placebo group for the next 96 hours. In this case, the trend of the results is the primary finding; the actual pain scores are not as critical as the relative differences between the NSAID and placebo groups.

7. **Tables:** Tables are appropriate when it is critical to present exact numerical values. However, not all results need be placed in either a table or figure. For example, the following table may not be necessary:Instead, the results could simply state that there was no inhibition of growth from 0.001-0.03% NaOCl, and a 100% inhibition of growth from 0.03-3% NaOCl (N=5/group). Similarly, if the results are not significant, then it is probably not necessary to include the results in either a table or as a figure. These and many other suggestions on figure and table construction are described in additional detail in Day (1998).

8. **Discussion:** This section should be used to interpret and explain the results. Both the strengths and weaknesses of the observations should be discussed. How do these findings compare to the published literature? What are the clinical implications? Although this last section might be tentative given the nature of a particular study, the authors should realize that even preliminary clinical implications might have value for the clinical leadership. Ideally, a review of the potential clinical significance is the last section of the discussion. What are the major conclusions of the study? How does the data support these conclusions

9. **Acknowledgments:** All authors must affirm that they have no financial affiliation (e.g., employment, direct payment, stock holdings, retainers, consultantships, patent licensing arrangements or honoraria), or involvement with any commercial organization with direct financial interest in the subject or materials discussed in this manuscript, nor have any such arrangements existed in the past three years. Any other potential conflict of interest should be disclosed. Any author for whom this statement is not true must append a paragraph to the manuscript that fully discloses any financial or other interest that poses a conflict. Likewise, the sources and correct attributions of all other grants, contracts or donations that funded the study must be disclosed

10. **References:** The reference style follows Index Medicus and can be easily learned from reading past issues of the JOE. The JOE uses the Vancouver reference style, which can be found in most citation management software products. Citations are placed in parentheses at the end of a sentence or at the end of a clause that requires a literature citation. Do not use superscript for references. Original reports are limited to 35 references. There are no limits to the number of references for review articles.

Manuscripts Category Classifications and Requirements

Manuscripts submitted to the *JOE* must fall into one of the following categories. The abstracts for all these categories would have a maximum word count of 250 words:

1. CONSORT Randomized Clinical Trial-Manuscripts in this category must strictly adhere to the Consolidated Standards of Reporting Trials-CONSORT-minimum guidelines for the publication of randomized clinical trials. These guidelines can be found at consort-statement.org. These manuscripts have a limit of 3,500 words, [including abstract, introduction, materials and methods, results, discussion, and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 4 figures and 4 tables*.
2. Review Article-Manuscripts in this category is either narrative articles, or systematic reviews/meta-analyses. Case report/Clinical Technique articles even when followed by the extensive review of the literature will be categorized as "Case Report/Clinical Technique". These manuscripts have a limit of 3,500 words, [including abstract, introduction, discussion, and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 4 figures and 4 tables*.
3. Clinical Research (*e.g.*, prospective or retrospective studies on patients or patient records, or research on biopsies, excluding the use of human teeth for technique studies). These manuscripts have a limit of 3,500 words [including abstract, introduction, materials and methods, results, discussion, and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 4 figures and 4 tables*.
4. Basic Research Biology (animal or culture studies on biological research on physiology, development, stem cell differentiation, inflammation or pathology). Manuscripts that have a primary focus on biology should be submitted in this category while manuscripts that have a primary focus on materials should be submitted in the Basic Research Technology category. For example, a study on cytotoxicity of a material should be submitted in the Basic Research Technology category, even if it was performed in animals with histological analyses. These manuscripts have a limit of 2,500 words [including abstract, introduction, materials

and methods, results, discussion, and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 4 figures or 4 tables*.

5. Basic Research Technology (Manuscripts submitted in this category focus primarily on research related to techniques and materials used, or with potential clinical use, in endodontics). These manuscripts have a limit of 2,500 words [including abstract, introduction, materials and methods, results, discussion, and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 3 figures and tables *.

6. Case Report/Clinical Technique (*e.g.*, report of an unusual clinical case or the use of cutting-edge technology in a clinical case). These manuscripts have a limit of 2,500 words [including abstract, introduction, materials and methods, results, discussion, and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 4 figures or tables*.* Figures, if submitted as multi-panel figures must not exceed 1-page length. Manuscripts submitted with more than the allowed number of figures or tables will require the approval of the JOE Editor or associate editors. If you are not sure whether your manuscript falls within one of the categories above, or would like to request preapproval for submission of additional figures please contact the Editor by email at jendodontics@uthscsa.edu.Importantly, adhering to the general writing methods described in these guidelines (and in the resources listed below) will help to reduce the size of the manuscript while maintaining its focus and significance. Authors are encouraged to focus on only the essential aspects of the study and to avoid inclusion of extraneous text and figures. The Editor may reject manuscripts that exceed these limitations.

Certificado do Comitê de Ética no uso de animais

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
FACULDADE DE MEDICINA VETERINÁRIA

CEUA - Comissão de Ética no Uso de Animais
CEUA - Ethics Committee on the Use of Animals

CERTIFICADO

Certificamos que o Projeto de Pesquisa intitulado **"Inter-relação entre a fibrose hepática e a periodontite apical"**, Processo FOA nº 00430-2018, sob responsabilidade de Luciano Tavares Angelo Cintra apresenta um protocolo experimental de acordo com os Princípios Éticos da Experimentação Animal e sua execução foi aprovada pela CEUA em 14 de Agosto de 2018.

VALIDADE DESTE CERTIFICADO: 14 de Julho de 2020.
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 14 de Agosto de 2020.

CERTIFICATE

We certify that the study entitled **"Interrelation between liver fibrosis and apical peridodontitis"**, Protocol FOA nº 00430-2018, under the supervision of Luciano Tavares Angelo Cintra presents an experimental protocol in accordance with the Ethical Principles of Animal Experimentation and its implementation was approved by CEUA on August 14, 2018.

VALIDITY OF THIS CERTIFICATE: July 14, 2020.
DATE OF SUBMISSION OF THE FINAL REPORT: August 14, 2020.

Prof. Ass. Dr. Leonardo Perez Faverani
Coordenador da CEUA
CEUA Coordinator

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