UNIVERSIDADE ESTADUAL PAULISTA
"Júlio de Mesquita Filho"

Isabela Joane Prado Silva

DISSERTAÇÃO DE MESTRADO

Influência da laserterapia de baixa intensidade no processo inflamatório e na imunomarcação da interleucina–23 e do fator indutor de hipóxia 1-alfa no tecido pulpar de dentes clareados

ARAÇATUBA - SP

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Orientadora: Profa. Francine Benetti

Coorientador: Prof. Luciano Tavares Angelo Cintra

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"Aqueles que passam por nós, não vão sós, não nos deixam sós. Deixam um pouco de si, e levam um pouco de nós. "

- Antoine de Saint-Exupéry.

“ Sem sonhos a vida é uma manhã sem orvalhos, um céu sem estrelas, um oceano sem ondas, uma vida sem aventura, uma existência sem sentido. ”

- Augusto Cury

Influência da laserterapia de baixa intensidade no processo inflamatório e na imunomarcação da interleucina–23 e do fator indutor de hipóxia 1-alfa no tecido pulpar de dentes clareados

Resumo

Introdução: O uso do laser infravermelho (LIV) através da fotobiomodulação, tem demonstrado efeitos benéficos aos tecidos. **Objetivos:** Avaliar a influência do LIV sobre o processo inflamatório, por meio da imunomarcação da interleucina pró-inflamatória IL-23, e sobre a angiogênese, por meio da imunomarcação do fator induzível por hipóxia-1 alfa (HIF-1α), no tecido pulpar de dentes clareados. **Materiais e métodos:** Quarenta ratos *Wistar* foram distribuídos aleatoriamente em 4 grupos de 20 hemi-maxilas cada: Grupo Controle – recebeu o tratamento com o gel placebo; Grupo Cla - recebeu 30 minutos do gel clareador H_2O_2 a 35%; Grupo LIV – recebeu uma aplicação de LIV (808 nm, 30 segundos, 3J); Grupo Cla-LIV – imediatamente após a aplicação do H_2O_2 a 35%, recebeu uma aplicação de LIV, como descrito no grupo LIV. Após 2 e 30 dias (n = 10), os animais foram eutanasiados e as maxilas removidas e processadas para avaliação histológica (H.E.) e imunoistoquímica. **Forma de análise:** Os cortes teciduais seriados e com espessura de 5 µm corados em H.E. foram avaliados por escores atribuídos à inflamação, e a análise imunoistoquímica foi realizada através de escores atribuídos à imunomarcação. Os dados foram submetidos aos testes de Wilcoxon e Mann Whitney ($p < 0,05$). **Resultados:** Aos 2 dias, houve inflamação severa e necrose nos terços oclusal e médio do tecido pulpar no grupo Cla, diferente do grupo Cla-LIV com inflamação leve à moderada ($p < 0,05$). No terço cervical, houve inflamação moderada a severa no grupo Cla, e leve no grupo Cla-LIV ($p < 0,05$). Aos 30 dias, houve ausência de inflamação e os grupos clareados apresentaram deposição de dentina terciária. Em relação à IL-23, aos 2 dias foi observada imunomarcação severa no grupo Cla e moderada no grupo Cla-LIV ($p < 0,05$); aos 30 dias, houve redução na imunomarcação de IL-23 nos grupos clareados, onde o grupo Cla apresentou imunomarcação moderada, e o grupo Cla-LIV leve imunomarcação, sem diferença significante ($p > 0,05$). HIF-1α foi mais presente aos 2 dias no grupo Cla, sem diferença significativa com Cla-LIV ($p > 0,05$). Foi observada diferença entre os grupos clareados e seus respectivos controles, que não apresentaram imunomarcação ($p < 0,05$); aos 30 dias, o grupo Cla apresentou redução na imunomarcação para HIF-1α, enquanto o grupo Cla-LIV apresentou aumento da imunomarcação, mas a diferença permaneceu apenas entre os grupos clareados e seus controles ($p > 0,05$). **Conclusão:** O laser infravermelho minimizou o infiltrado inflamatório e a imunomarcação de IL-23 no tecido pulpar após a clareação dentária, mas não influenciou a imunomarcação de HIF-1α.

Palavras-chave: clareação dentária, interleucina-23, fator induzível por hipóxia-1 alfa, angiogênese, inflamação pulpar, laser infravermelho

Influence of low-level laser therapy on the inflammatory process and immunolabeling of interleukin-23 and hypoxia-inducing factor 1-alpha in the pulp tissue of bleached teeth

Abstract

Introduction: The use of infrared laser (IRL) through photobiomodulation has shown beneficial effects on tissues. **Objectives**: To evaluate the influence of LLL on the inflammatory process, by immunolabeling IL-23 pro-inflammatory interleukin, and on angiogenesis, by immunolabeling hif-1 alpha inducible factor (HIF-1α) in the pulp tissue of bleached teeth (HIF-1α). **Materials and methods**: Forty *Wistar* rats were randomly divided into 4 groups of 20 hemimaxilla each: control group - received treatment with placebo gel; Ble group - received 30 minutes of 35% H_2O_2 bleaching gel; IRL group - received one application of IRL (808 nm, 30 seconds, 3 J); Ble-IRL group - immediately after application of 35% H_2O_2, received an application of IRL, as described in the IRL group.. After 2 and 30 days (n = 10), the rats were euthanized and the jaws removed and processed for histological evaluation (H.E.) and immunohistochemistry. **Form of analysis**: Serial tissue sections with thickness of 5 µm stained in H.E. were evaluated by scores attributed to inflammation, and immunohistochemical analysis was performed by scores attributed to immunolabeling. The data were submitted to the Wilcoxon and Mann Whitney tests ($P < 0.05$). **Results:** At 2 days, there was a severe inflammation and the presence of necrosis in the occlusal and middle thirds of the pulp tissue in the Ble group, different compared to the Ble-IRL group with moderate to mild inflammation ($P < 0.05$). In the cervical third, there was moderate to severe inflammation in the Ble group, and mild in the Ble-IRL group ($P < 0.05$). At 30 days, there was absence of inflammation and bleached groups had an extensive deposition of tertiary dentin. Regarding IL-23, at 2 days was observed severe immunolabeling in the Ble group and moderate in the Ble-IRL group ($P < 0.05$); at 30 days, there was a reduction in IL-23 immunolabeling in the bleached groups, where Ble group had moderate immunolabeling, and Ble-IRL group had mild immunolabeling, without significant difference ($P > 0.05$). HIF-1α was more evident at 2 days in the Ble group, without significant difference with Ble-IRL ($P > 0.05$). The difference was observed between the bleached groups and their respective controls, which had no immunolabeling ($P < 0.05$); at 30 days, the Ble group had a reduction in HIF-1α immunolabeling, while the Ble-IRL group had an increase in immunolabeling from moderate to severe; however, the difference remained only between the bleached groups and their controls ($P > 0.05$). **Conclusion:** Infrared laser minimized the inflammatory infiltrate and IL-23 immunolabeling in the pulp tissue after dental bleaching, but did not influenced HIF-1α immunolabeling.

Keywords: dental bleaching, interleukin-23, hypoxia-inducible factor-1α, angiogenesis, pulp inflammation, infrared laser.

SUMÁRIO

Páginas

I. Introdução

A clareação dentária é um dos tratamentos estéticos mais utilizados na odontologia contemporânea, devido ao seu elevado índice de sucesso clínico, diretamente relacionado à capacidade do peróxido de hidrogênio (H_2O_2) em se difundir por esmalte e dentina (Briso *et al*. 2014, 2015, Cintra *et al*. 2016a). O H_2O_2 é o componente ativo do gel utilizado em procedimentos de clareação dentária, onde ocorre oxidação de estruturas orgânicas por meio da ação de espécies reativas de oxigênio (EROs) (Eimar *et al*. 2012).

Devido à capacidade das EROs em penetrar nos tecidos dentários mineralizados, estas atingem a polpa coronária e ocasionam efeitos imediatos e com consequência a longo prazo (Briso *et al*. 2015, Cintra *et al*. 2016a). Dentre os danos ao tecido pulpar, foram observados processo inflamatório acentuado, áreas de necrose, deposição de dentina terciária, e expressão de marcadores da mineralização (Cintra *et al*. 2016b, Benetti *et al*. 2017, 2018b, 2019a, 2019b). Como consequência, a maior parte dos pacientes relatam sensibilidade dentária após a realização do procedimento clareador (Pintado-Palomino *et al*. 2015, Rahal *et al*. 2018).

Diversos protocolos têm sido descritos a fim de minimizar os efeitos dos géis clareadores em dentes vitais, com variações na concentração e no período de aplicação do gel (Almeida *et al*. 2015, Cintra *et al*. 2016b, Benetti *et al*. 2017, 2018b). É importante que estes protocolos mantenham a capacidade clareadora, mas sem provocar alterações que sejam negativas ao tecido pulpar (Soares *et al*. 2014, Cintra *et al*. 2016a).

Protocolos modificados com diferentes agentes terapêuticos também foram propostos. Dentre eles, o uso de agentes antioxidantes ou anti-inflamatórios de uso sistêmico, simultaneamente à clareação dentária (Charakorn *et al.* 2009, Sasaki *et al*. 2009, May *et al.* 2010, Vargas *et al*. 2014, Faria-E-Silva *et al*. 2015). Porém, ainda não foi encontrado um tratamento completamente eficaz.

Em estudos recentes, verificamos que a aplicação tópica de anti-inflamatório com ação corticosteroide alcançou resultados positivos na minimização da inflamação tecidual da polpa de dentes clareados (Benetti *et al*. 2018a, Gallinari *et al*. 2019). No entanto, uma terapia que pudesse atuar não

apenas para minimizar o processo inflamatório, mas ainda, favorecesse a angiogênese e o processo regenerativo, seria o ideal.

Diversos protocolos de uso do laser de baixa intensidade (LBI) também foram avaliados para minimizar os danos do gel clareador à polpa dentária (Dantas *et al*. 2010, Lima *et al*. 2014, Benetti *et al*. 2018c), devido seus efeitos bioestimulantes, além de ação analgésica e anti-inflamatória (Reddy 2004, Silveira *et al.* 2007, Ladalardo *et al.* 2004, Moosavi *et al.* 2016). Estudos realizados com o LBI apontaram resultados benéficos aos tecidos, pelo aumento da síntese de trifosfato de adenosina (ATP) (Robertson & Melfi 1980, Karu 1999), e da síntese proteica, ambos capazes de atuar no reparo tecidual (Loevshchall & Arenholt-Bindslev 1994, Yu *et al.* 1996). Ainda, LBI resultou em elevada expressão de enzimas que atuam minimizando o estresse oxidativo (Polo *et al.* 1999, Jori *et al.* 1996, Silveira *et al.* 2007), o que seria de grande importância para o tecido pulpar que entrou após a ação do H_2O_2 do gel clareador.

Para que o LBI tenha seu efeito esperado, se faz necessária uma avaliação das condições de absorção do tecido que irá receber sua aplicação (American Academy of Periodontology, 2002). Os efeitos primários do LBI ocorrem em nível celular e/ou molecular, gerando posteriormente, os efeitos secundários destes fenômenos, como o aumento da síntese de colágeno e da angiogênese (Karu 1989)

No entanto, um protocolo eficaz e seguro para o uso do LBI na clínica ainda não foi determinado. Na presente proposta, trabalhamos com LBI utilizando laser infravermelho (LIV) com os parâmetros de 808 nm, 3 J e 100 mW, com densidade energética de 100 J/cm^2, em aplicação única de 30 segundos imediatamente após a sessão clareadora. Estes parâmetros apresentaram resultados positivos em estimular a recuperação do tecido pulpar após procedimento clareador em dentes de ratos, em um estudo realizado por nosso grupo de pesquisa (Figura 1; Terayama *et al*. 2020 *in press*).

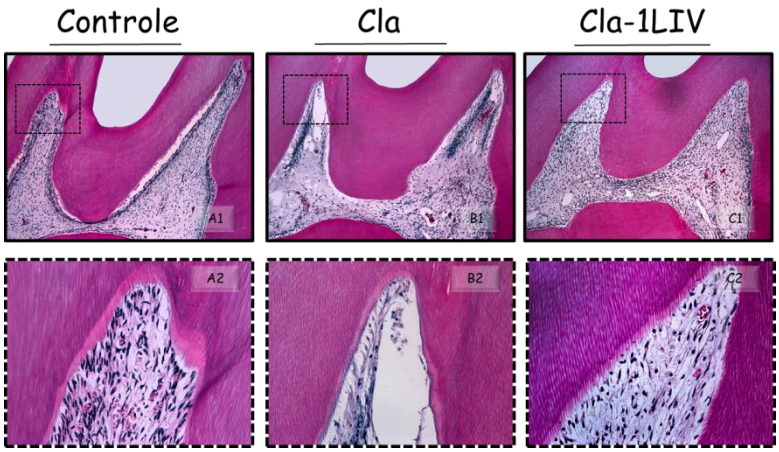

Figura 1. Imagens representativas dos grupos aos 2 dias após sessão clareadora. Grupo Controle com (A1) aspecto da polpa coronária evidenciando tecido pulpar em normalidade e (A2) corno pulpar com camada de odontoblastos organizada e ausência de inflamação. Grupo Clareado (Cla) (B1) onde pode ser observada necrose, infiltrado inflamatório severo e desorganização tecidual e (B2) corno pulpar evidenciando necrose na região. Grupo Cla seguido de uma aplicação de LIV (Cla-1LIV) (C1) com desorganização tecidual e presença de inflamação e (C2) corno pulpar evidenciando presença de células inflamatórias em meio ao tecido pulpar desorganizado, mas ausência de necrose. [100X, 400X - H.E.]

Algumas citocinas, como fator de necrose tumoral (TNF)-α, interleucina (IL)-6, e IL-17 participam do processo inflamatório do tecido pulpar após clareação dentária (Benetti *et al*. 2018b, Ferreira *et al*. 2018). Ainda, observamos que o aumento da concentração de H_2O_2 é acompanhado da ativação prolongada de células CD5-positivas, um receptor presente em células semelhantes a linfócitos, mesmo após 30 dias do procedimento clareador, quando o tecido pulpar já está organizado (Benetti *et al*. 2018b). Assim, ainda há o que se esclarecer sobre a resposta inflamatória gerada na polpa dentária após longos períodos do procedimento clareador, e a influência que a LBI pode exercer nesse processo.

Para ampliar nossos estudos, podemos lançar mão de outras citocinas importantes observadas anteriormente em tecido pulpar inflamado, tal como a IL-23 (Lu *et al*. 2002, Silva *et al*. 2009). Isso porque o conjunto IL-23 e IL-17 parece ter um papel crítico em processos inflamatórios (Iwakura & Ishigame 2006, Ohyama *et al*. 2009). A IL-17 já foi identificada por nós no tecido pulpar de molares de ratos após clareação dentária (Benetti *et al*. 2018b, Ferreira *et al*. 2018). Dado a presença de IL-17 na inflamação da polpa dentária após este

procedimento, a IL-23 pode desempenhar um papel importante neste processo inflamatório.

Alterações vasculares também possuem impacto sobre o metabolismo pulpar (Derringer & Linden 2004, Domínguez *et al*. 2013). A angiogênese consiste na formação de novas estruturas capilares (Hamilton & Gutmann 1999), e foi mostrado que o LBI tem efeito importante para favorecer este processo (Abi-Ramia *et al*. 2010, Colombo *et al*. 2013). Hiperemia transitória na polpa foi observada após a aplicação do LBI durante o movimento ortodôntico, e relacionada a um reparo acelerado do tecido pulpar (Abi-Ramia *et al*. 2010). Também se observou que a angiogênese em feridas cutâneas no dorso de ratos foi mais intensa após tratamento com LBI (Colombo *et al*. 2013). Embora a permeabilidade vascular seja aumentada em tecido pulpar de dentes de ratos clareados (Ferreira *et al*. 2013), a formação de novos vasos sanguíneos ainda não foi avaliada. Apesar dos estudos demonstrarem efeito do LBI na angiogênese, pouco se sabe sobre os efeitos do H_2O_2 neste mecanismo celular.

Uma serie de moléculas são envolvidas no processo angiogênico. O fator induzível por hipóxia-1 alfa (HIF-1α) é considerado o principal regulador na transição da resposta celular e desenvolvimento da hipóxia, no qual através desta, potencializa as propriedades terapêuticas das células-tronco mesenquimais (Cerrada *et al*. 2013). Com função de interação com genes relacionados à angiogênese e eritropoiese, caracteriza-se por estimular à síntese de colágeno com consequente regulação do ciclo celular, favorecendo a sobrevivência e melhoria da resistência natural da célula (Schodel *et al*. 2010).

Desta forma, o presente estudo teve como objetivo investigar a influência do LBI sobre os danos causados pelo gel clareador no tecido pulpar, avaliando o infiltrado inflamatório, e a imunomarcação de IL-23 e HIF-1α, no tecido pulpar de dentes clareados. Anteriormente, obtivemos resultados positivos com aplicação única do LIV após a clareação dentária (Terayama *et al*. 2018), com redução do processo inflamatório gerado pelo gel clareador à base de H_2O_2 a 35%. Assim, este foi o protocolo de escolha para o presente trabalho.

II. Artigo

International Endodontic Journal

Low-level laser therapy reduces inflammation caused for hydrogen peroxide but not influences in the immunolabeling of hypoxia-inducible factor-1α in pulp tissue after bleaching

Abstract

Aim To evaluate the influence of low-level laser therapy (LLLT) through the use of infrared laser (IRL) in the pulp tissue of rat molars after bleaching, regarding the inflammation and immunolabeling of interleukin (IL)-23 and hypoxia-inducible factor (HIF)-1α.

Methodology The maxillary molars of forty rats were divided into 4 groups of 20 hemi-maxilla each: Control – received treatment with placebo gel; Bleached (Ble) group – received 30 min of the 35% hydrogen peroxide (H_2O_2) bleaching gel; IRL group – received one application of the IRL (808 nm, 30 sec, 3 J); Ble-IRL group – immediately after the application of 35% H_2O_2, received one application of the IRL, as described in the IRL group. After 2 and 30 days (n = 10), the rats were euthanized and the pulp tissue was evaluated using inflammation and immunolabeling scores. The data were submitted to the Wilcoxon and Mann-Whitney tests ($P<0.05$).

Results At 2 days, there was a severe inflammation and necrosis in the occlusal and middle thirds of the pulp tissue in the Ble group, different to the Ble-IRL group with moderate to mild inflammation ($P<0.05$); in cervical third, there was moderate to severe inflammation in the Ble group, and mild in the Ble-IRL ($P<0.05$). At 30 days, there was absence of inflammation and bleached groups had extensive deposition of tertiary dentin. Regarding IL-23, at 2 days was observed severe immunolabeling in Ble group compared to the Ble-IRL, which had moderate immunolabeling ($P<0.05$); at 30 days, there was a reduction in IL-23 immunolabeling in the bleached groups, where Ble had moderate immunolabeling, and Ble-IRL group had mild immunolabeling ($P>0.05$). HIF-1α was more evident at 2 days in the Ble group, without significant difference with Ble-IRL ($P>0.05$). The difference was observed between the bleached groups and their respective controls, without immunolabeling ($P<0.05$); at 30 days, the Ble group had a reduction in HIF-1α immunolabeling, while the Ble-IRL had an increase in immunolabeling; however, the difference remained only between the bleached groups and their controls ($P>0.05$).

Conclusion LLLT, through the use of IRL, minimized the inflammatory infiltrate and IL-23 immediate immunolabeling in the pulp tissue after dental bleaching, but not influenced the immunolabeling of HIF-1α.

Introduction

During dental bleaching, the oxidation of organic structures of dental tissue occurs through the action of reactive oxygen species (ROS) released from hydrogen peroxide (H_2O_2) (Eimar *et al*. 2012). Due to the ability of ROS to penetrate through mineralized dental tissues, they reach the dental pulp (Briso *et al*. 2015, Cintra *et al*. 2016a), and may cause damage such as severe and late inflammatory process, areas of necrosis, subsequent deposition of mineralized tissue, and pulp aging (Cintra *et al*. 2016b, 2017, Benetti *et al*. 2017, 2018b, 2019a, 2019b). Clinically, the response to pulp damage is observed by the intense tooth sensitivity reported by patients in the initial periods after the procedure (Pintado Palomino *et al*. 2015, Rahal *et al*. 2018).

Some protocols have been described to minimizing the effects of bleaching on vital teeth, such as variations in the H_2O_2 concentration and time of application of the gel (Almeida *et al*. 2015, Cintra *et al*. 2016b, Benetti *et al*. 2017, 2018b). Low-level laser therapy (LLLT) was also evaluated (Dantas *et al*. 2010, Lima *et al*. 2014, Benetti *et al*. 2018c) with the justification of its biostimulant effects, as well as analgesic and anti-inflammatory action (Ladalardo *et al*. 2004, Reddy 2004, Silveira *et al*. 2007, Moosavi *et al*. 2016). Studies showed beneficial results of LLLT to different tissues by increasing adenosine triphosphate synthesis (Robertson & Melfi 1980, Karu 1999) and protein synthesis, both able to act in tissue repair (Loevshchall & Arenholt-Bindslev 1994, Yu *et al*. 1996). In addition, the expression of enzymes that act minimizing oxidative stress was high (Jori *et al*. 1996, Polo *et al*. 1999, Silveira *et al*. 2007).

Previously, it was observed that some cytokines, such as tumor necrosis factor (TNF) - α, interleukin (IL)-6 and IL-17, participate in the pulp tissue inflammatory process after tooth bleaching (Benetti *et al*. 2018b, Ferreira *et al*. 2018). The increased concentration of H_2O_2 is accompanied by prolonged activation of CD5-positive cells, a receptor present in lymphocyte-like cells, even 30 days after the bleaching, when the pulp tissue is already organized (Benetti *et al*. 2018b). The expression of these cytokines after bleaching can be reduced after some therapies (Benetti *et al*. 2018a, Gallinari *et al*. 2019).

Thus, other important cytokine previously observed in inflamed pulp tissue, the IL-23 (Lu *et al*. 2002, Silva *et al*. 2009), could indicate if LLLT would be able to act in this tissue. The combination IL-23 and IL-17 plays a critical role in inflammatory processes (Iwakura & Ishigame 2006, Ohyama *et al*. 2009), and IL-17 was present in the pulp inflammation after dental bleaching (Benetti *et al*. 2018a, 2018b, Ferreira *et al*.

2018). In this way, IL-23 may be present in the pulp tissue of bleached teeth. In addition, LLLT reduced IL-23 expression in epidermal treatment (Rácz *et al*. 2010), evidencing that the laser is able to act in the regulation of this interleukin.

ROS have also been associated with hypoxic microenvironments (Zepeda *et al*. 2013). The molecular response to hypoxia requires rapid action mechanisms that act within partial pressures of oxygen (O_2), and lead to an adaptive response with the activation of transcription factors, as an attempt to regulate tissue O_2 supply and energy metabolism (Zepeda *et al*. 2013).

The hypoxia-inducible factor (HIF)-1α transcription factor is a critical mediator of these adaptive responses, and is considered the main regulator in the transition of cellular response and development of hypoxia, which through this induces the therapeutic properties of mesenchymal stem cells (Cerrada *et al*. 2013). With function of interaction with genes related to angiogenesis, HIF-1α is characterized by stimulating collagen synthesis with consequent regulation of the cell cycle favouring the survival and improvement of natural cell resistance (Schodel *et al*. 2010).

Angiogenesis is the formation of new capillary structures (Hamilton & Gutmann 1999), and LLLT has an important effect on this process (Abi-Ramia *et al*. 2010, Colombo *et al*. 2013). Transient hyperemia in the pulp was observed after LLLT application during orthodontic movement, and related to accelerated pulp tissue repair (Abi-Ramia *et al*. 2010). It was also observed that angiogenesis on dorsal wound of rats was more intense after treatment with LLLT (Colombo *et al*. 2013). Although vascular permeability is increased in pulp tissue of bleached rat teeth (Ferreira *et al*. 2013), the angiogenesis process in these teeth has not yet been evaluated.

Thus, the aim of this study was to evaluate the influence of LLLT on pulp inflammation of bleached teeth through the analysis of the inflammatory infiltrate and the immunolabeling of pro-inflammatory cytokine IL-23. Furthermore, this study evaluated the presence of HIF-1α in the pulp tissue of these teeth, and the influence of LLLT on the immunolabeling of this transcription factor. The null hypothesis that LLLT does not influence on inflammatory process and in the HIF-1α immunolabeling in the pulp tissue of bleached teeth was adopted.

Materials and Methods

Animals

Forty 2-month-old male Wistar albino rats (weighing approximately 280 g) were used. The sample size was established on the basis of the findings of previous studies

(Benetti *et al*. 2019a, 2019b). The animals were maintained in a temperature-controlled environment (22°C ± 1°C, 70% humidity, and a 12-h light–dark cycle) and received water and food *ad libitum*. All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD, USA). The experimental protocol (CEUA-00713-2018) was approved by the local Ethics Committee.

Dental bleaching and LLLT treatment

The animals were anaesthetised by intramuscular injections of ketamine (80 mg/kg, Ketamina Agener 10%; União Química Farmacêutica Nacional S/A, Embu-Guaçu, São Paulo, Brazil) and xylazine (10 mg/kg, Xilazin; Syntec do Brasil LTDA, Cotia, São Paulo, Brazil). After the application and photo-activation of the resinous gingival barrier (Top Dam; FGM Dental Products, Joinville, SC, Brazil), the right or left maxillary molars of the rats were randomly treated (Table 1) with either bleaching gel (0.01 mL; single application of 30 min of the 35% H_2O_2; Whiteness HP Maxx; FGM Dental Products, Joinville, SC, Brazil) or placebo gel (0.01 mL; single application of 30 min of the thickener of the bleaching gel) (Cintra *et al*. 2016b). The right or left maxillary molars of each rat were randomized by lottery for application of treatment also defined by lottery. After bleaching, the tooth surface was wiped with cotton and absorbent paper and rinsed thoroughly with water. To standardize the applied volume of the bleaching gel, 1.0 mL syringes were used (Benetti *et al*. 2018b).

LLLT was carried according Terayama *et al*. (2020, *in press*) in half of the animals. Therefore, infrared laser (IRL) duo semiconductor (GaA1As; MM Optics Ltda, São Carlos, SP, Brazil) with a wavelength of 808 nm was used immediately after bleaching, during 30 seconds. The device had a fixed power of 100 mW. The beam exit area at the tip of the laser pen was 3 mm^2. The tip of the laser pen was kept in contact with the first molar (Dantas *et al*. 2010, Moosavi *et al*. 2016).

Thus, a split-mouth design was established after the bleaching and LLLT treatments, resulting in 4 experimental groups with 20 hemi-maxillae each: Control, bleached (Ble), IRL, and bleached-IRL (Ble-IRL).

Table 1 Distribution of experimental groups according to the treatment performed

Groups	Dental Bleaching	LLLT			
		nm	Time	J	Session
Control	-----	-----	-----	-----	-----
Ble	1 application of 30 min of the bleaching gel	-----	-----	-----	-----
IRL	-----	808	30 sec	3	Immediate
Ble-IRL	1 application of 30 min of the bleaching gel	808	30 sec	3	Immediate

Ble: bleached; IRL: infrared laser; J: joules; sec: second.

Histological analysis

At 2 days and at 30 days (Cintra *et al*. 2017, Benetti *et al*. 2018b, Ferreira *et al*. 2018), the animals were euthanized with an overdose of sodium thiopental (240 mg/kg; Thipentax, Cristália, Produtos Químicos Farmacêuticos LTDA, Itapira, SP, Brazil). The hemi-maxillae were separated, dissected, and fixed in a solution of 4% buffered formaldehyde (24 hours). The specimens were decalcified in a 10% ethylenediaminetetraacetic acid (3 months) and then dehydrated, clarified, and embedded in paraffin. Serial histological sections of each specimen were selected from the point where the mesial root of the first molar was at its full longitudinal extension. Subsequently, 5-µm sections were cut in the bucco-lingual plane. The blades with histological sections were then stained with haematoxylin-eosin for analysis using light microscopy (×400 magnification; DM4000 B; Leica Microsystems, Wetzlar, Germany). For each specimen were used 2 slides with 3 tissue sections each. The pulp chamber was divided by occlusal, middle and cervical thirds (Cintra *et al*. 2013) and the analysis of the inflammatory infiltrate was scored as follows: 0, inflammatory cells absent or negligible in number; 1, mild inflammatory infiltrate (< 25 cells per field); 2, moderate inflammatory infiltrate (between 25 and 125 cells per field); 3, severe inflammatory infiltrate (> 125 cells per field); and 4, tissue necrosis (Benetti *et al*. 2019a, 2019b, Louzada *et al*. 2019). The analysis was performed by a single calibrated operator in a blinded manner.

Immunohistochemical analyses

Other histological sections of groups were obtained for immunohistochemical assessments with an indirect immunoperoxidase technique (Cintra *et al*. 2017, Benetti *et al*. 2018a) for IL-23 and HIF-1α. The histological sections were deparaffinised in xylene and hydrated in a decreasing ethanol series. Antigen retrieval was performed by immersing the histological slides in buffer citrate solution (Antigen Retrieval Buffer; Spring Bioscience, Pleasanton, CA, USA) in a pressurised chamber (Decloaking Chamber; Biocare Medical, Concord, CA, USA) at 95°C for 10 min. The slides were rinsed with phosphate-buffered saline at the end of each stage of the immunohistochemical reaction. The histological sections were immersed in 3% H_2O_2 solution for 1 h and 20 min and in 1% bovine serum albumin for 12 h to block the endogenous peroxidase activity and nonspecific sites, respectively. The histological slides were divided and incubated with one of the following primary antibodies: anti-HIF-1α and anti-IL-23 (rabbit primary antibodies; Sigma-Aldrich Co. LLC, St. Louis, MO, USA). The primary antibodies were diluted (Antibody Diluent with Background Reducing Components; Dako Laboratories, Carpinteria, CA, USA), and placed in a moist chamber for 24 h. Then, the histological sections were incubated with a biotinylated secondary antibody for 1 h and 30 min and subsequently treated with streptavidin–horseradish peroxidase conjugate for 1 h and 30 min (Universal Dako Labelled Streptavidin-Biotin kit; Dako Laboratories). After rinsing with PBS, the reaction was developed using the chromogen 3,3′-diaminobenzidine tetrahydrochloride (DAB Chromogen kit; Dako Laboratories) and counterstained with haematoxylin. The negative controls consisted of specimens that underwent the previously described procedures without treatment with the primary antibodies.

For analyses, the immunolabelling was defined as the presence of a brownish colour in the cytoplasm of the cells and extracellular matrix. Because immunolabelling of both the cells and the extracellular matrix is of great importance for study, a semi-quantitative analysis was performed, which provides information on the numbers of immunolabeled cells and immunolabelling intensity of the extracellular matrix (Ferreira *et al*. 2018, Benetti *et al*. 2019a). The scores were assigned as follows (Ferreira *et al*. 2018, Benetti *et al*. 2019a): 0, immunolabelling missing (absence of labeling in extracellular matrix and complete absence of immunoreactive cells); 1, low pattern of immunolabelling (weak labeling of the extracellular matrix and approximately one quarter of the immunoreactive cells); 2, moderate pattern of immunolabelling (moderate

labeling of the extracellular matrix and approximately one half of the immunoreactive cells); 3, severe pattern of immunolabelling (strong labeling of the extracellular matrix and approximately three quarters of the immunoreactive cells); and 4, very severe pattern of immunolabelling (extremely strong labeling of the extracellular matrix and approximately all immunoreactive cells). Data were collected and analysed by a single-calibrated, blinded operator.

Statistical analyses

Wilcoxon signed-rank test and Mann-Whitney test were used for statistical comparisons of pulp damage, inflammatory response, and immunohistochemistry at the significance level of 5% (Sigma-Plot 12.0, $P < 0.05$).

Results

Analysis of the inflammatory infiltrate

The representative images of the inflammatory infiltrate analysis at 2 and 30 days are showed in Figure 1, and the results are described in Table 2. At 2 days, it was possible to observe a higher amount of inflammatory cells and the presence of necrosis in the occlusal and middle thirds of the pulp tissue in the Ble group when compared to group to Ble-IRL group, which showed lesser inflammatory infiltrate and absence of necrosis ($P < 0.05$). In the cervical third, there was moderate to severe inflammatory infiltrate in the Ble group, and mild inflammatory infiltrate in the Ble-IRL group, with a significant difference between both groups ($P < 0.05$).

The inflammation and necrosis observed in Ble group was significantly different compared to the control group, which showed no inflammation in all thirds of the pulp chamber ($P < 0.05$). In contrast, the inflammation observed in the Ble-IRL group was significantly different compared to the IRL group, which showed no inflammation, only in the occlusal and middle thirds ($P < 0.05$); in the cervical third, there was no significant difference between the Ble-IRL and IRL groups ($P > 0.05$).

At 30 days, all groups had pulp tissue with cellular organization, presence of odontoblastic layer, and absence of inflammatory cells. Both bleached groups had an extensive deposition of tertiary dentin.

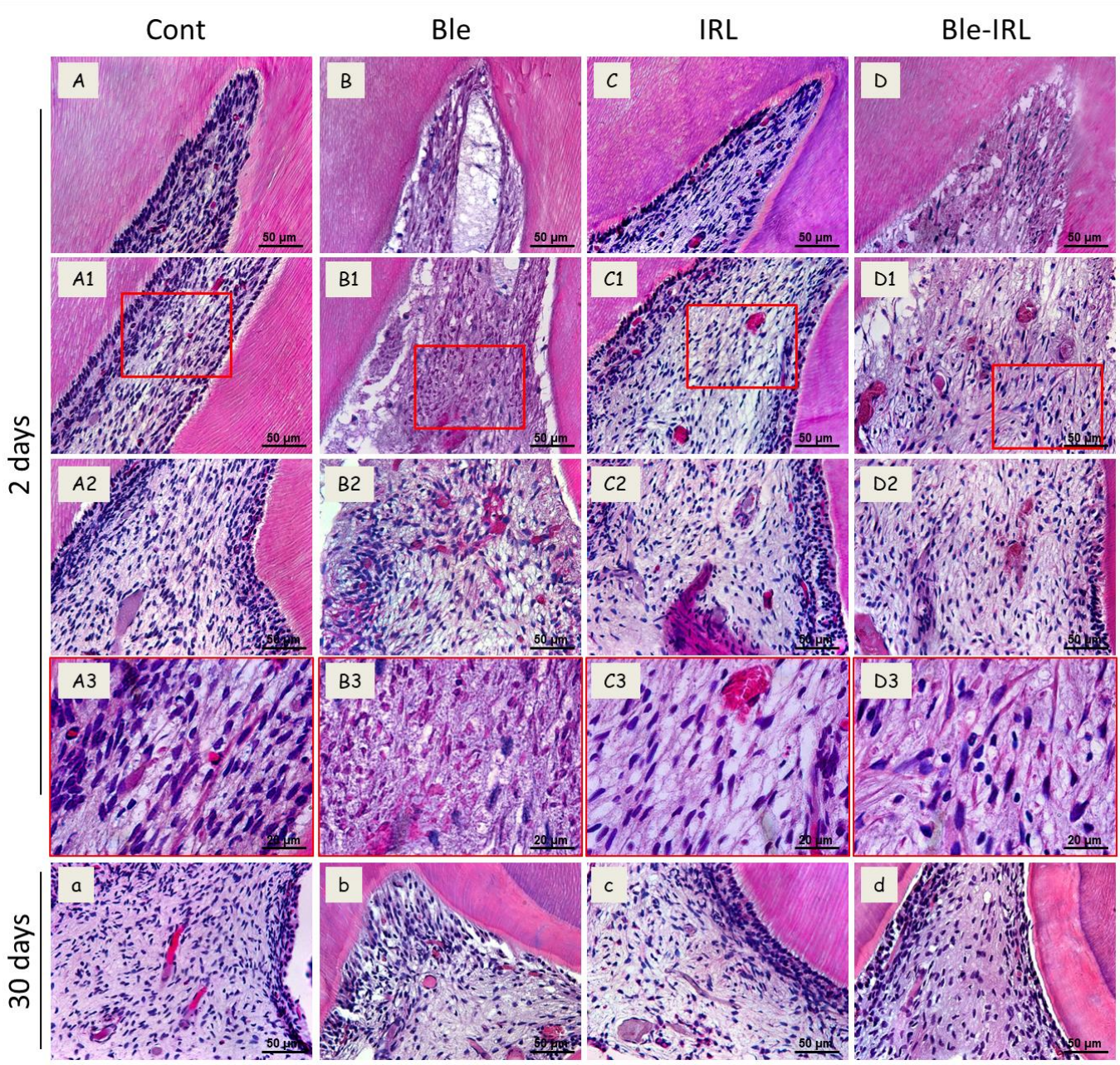

Figure 1 Representative images of the inflammation analysis of the groups at 2 and 30 days. Images of (A-D) occlusal, (A1-D1) middle and (A2-D2) cervical thirds of groups, and (A3-D3) higher magnification to evidencing pulp tissue of each group, at 2 days. (A-A3) Control group without alteration and absence of inflammation; (B-B3) Ble group with evident necrosis area and severe inflammatory infiltrate; (C-C3) IRL group with organised pulp tissue and absence of inflammation; and (D-D3) Ble-IRL group with moderate to mild inflammation. (a-d) Images of (a) control, (b) Ble, (c) IRL and (d) Ble-IRL groups at 30 days, evidencing absence of inflammation and presence of tertiary dentin in bleached groups. [400×, 1000×; H.E.]

Table 2 Attributed scores to inflammatory infiltrate in each group

Analysis		Scores	Groups				*P* value
			Cont	Ble	IRL	Ble-IRL	
H.E.	**Occlusal**	0	10/10	0/10	10/10	0/10	*Cont×Ble: = 0.002 *IRL×Ble-IRL: = 0.002 *Ble×Ble-IRL: < 0.001
		1	0/10	0/10	0/10	0/10	
		2	0/10	0/10	0/10	6/10	
		3	0/10	4/10	0/10	4/10	
		4	0/10	6/10	0/10	0/10	
		Median	0	4	0	2	
	Middle	0	10/10	0/10	10/10	0/10	*Cont×Ble: = 0.002 *IRL×Ble-IRL: = 0.002 *Ble×Ble-IRL: < 0.001
		1	0/10	0/10	0/10	4/10	
		2	0/10	3/10	0/10	6/10	
		3	0/10	7/10	0/10	0/10	
		4	0/10	0/10	0/10	0/10	
		Median	0	3	0	2	
	Cervical	0	10/10	0/10	10/10	5/10	*Cont×Ble: = 0.002 IRL×Ble-IRL: = 0.063 *Ble×Ble-IRL: < 0.001
		1	0/10	0/10	0/10	5/10	
		2	0/10	6/10	0/10	0/10	
		3	0/10	4/10	0/10	0/10	
		4	0/10	0/10	0/10	0/10	
		Median	0	2	0	1	

Cont×Ble and IRL×Ble-IRL = Wilcoxon test ($P < 0.05$);

Ble×Ble-IRL and Cont×IRL = Mann-Whitney test ($P < 0.05$).

*The symbol highlights the significant difference among groups.

Immunohistochemical Analyses

Representative images of the immunohistochemical analyses are shown in Figure 2, and the results are shown in Table 3. Regarding the immunolabeling of IL-23, at 2 days was observed higher immunolabeling in the Ble group, which had severe immunolabeling compared to the control group, with no immunlabeling in most specimens ($P < 0.05$); moreover, the Ble group differed significantly from the Ble-IRL group, which had moderate immunolabeling ($P < 0.05$). The IRL group had no immunolabeling, unlike the Ble-IRL group ($P < 0.05$), and similar to the control group ($P > 0.05$).

At 30 days, there was a reduction in IL-23 immunolabeling in the bleached groups, where Ble group had moderate immunolabeling, and Ble-IRL group had mild

immunolabeling, but with no significant difference between both ($P > 0.05$). The difference remained between the bleached groups and their respective controls ($P < 0.05$).

Regarding the immunohistochemical analysis of HIF-1α, the immunolabeling was more evident at 2 days in the Ble group, with a severe immunolabeling in most specimens, while the Ble-IRL group had moderate immunolabeling; however, there was no significant difference in both groups ($P > 0.05$). The difference was observed between the bleached groups and their respective controls, which had no immunolabeling ($P < 0.05$).

At 30 days, the Ble group had a reduction in HIF-1α immunolabeling, while the Ble-IRL group had an increase in immunolabeling from moderate to severe; however, the difference remained only between the bleached groups and their controls ($P > 0.05$).

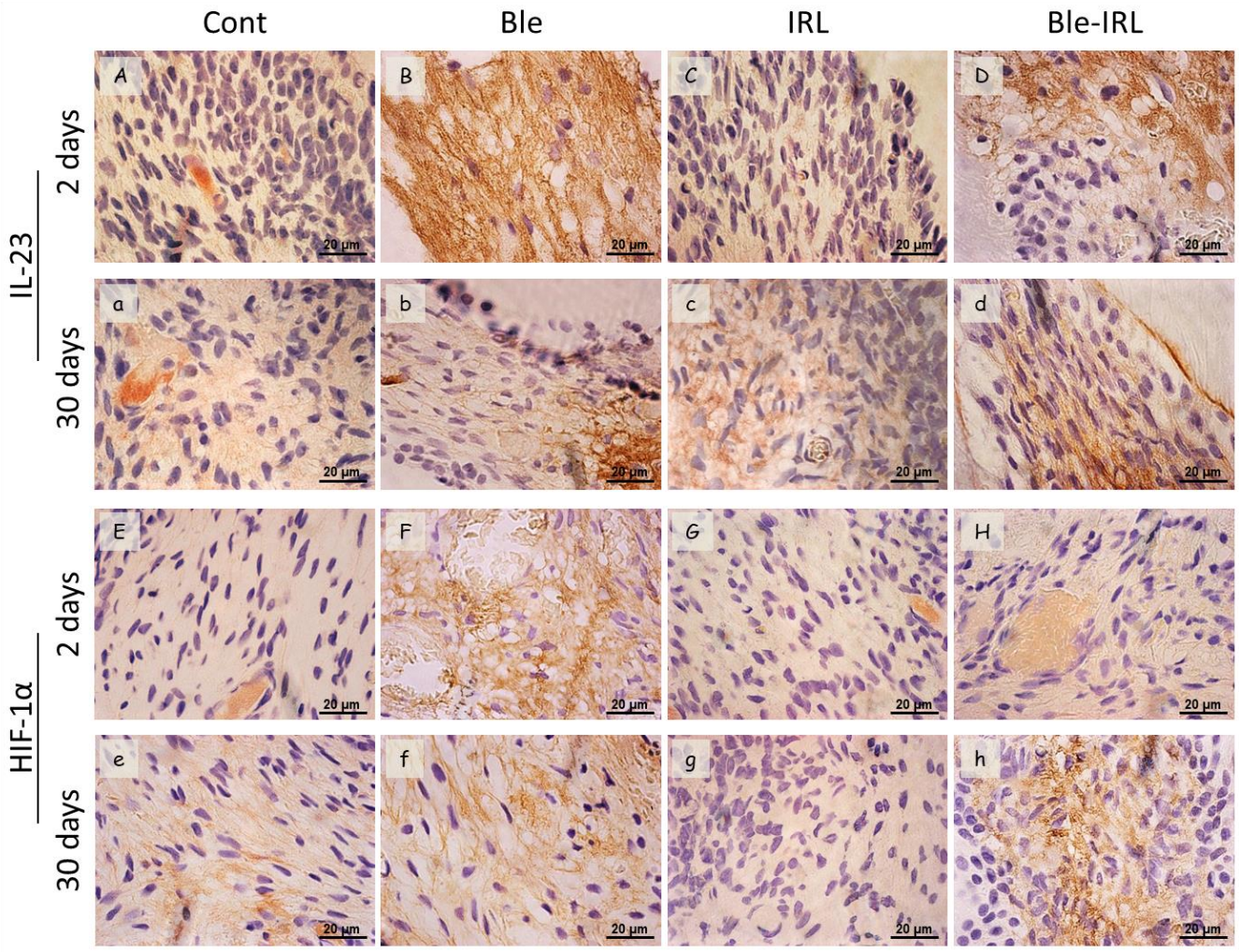

Figure 2 Representative images of the immunolabeling of IL-23 and HIF-1α in each group, at 2 and 30 days. Images of (A,a) control and (C,c) IRL groups with absence of IL-23 immunolabeling at (A, C) 2 and (a, c) 30 days; (B,b) Ble group with severe and moderate immunolabeling at (B) 2 and (b) 30 days, respectively; and (D,d) Ble-IRL group with moderate and mild immunolabeling at (D) 2 and (d) 30 days, respectively. Images of (E,e) control and (G,g) IRL groups with absence of HIF-1α immunolabeling at (E, G) 2 and (e, g) 30 days; (F,f) Ble group with moderate and mild immunolabeling at (F) 2 and (f) 30 days, respectively; and (H,h) Ble-IRL group with mild and moderate immunolabeling at (H) 2 and (h) 30 days, respectively. [1000×; immunohistochemical]

Table 3 Attributed scores to immunolabeling of IL-23 and HIF-1α in each group

Analysis		Scores	Groups				*P*
			Cont	Ble	IRL	Ble-IRL	
IL-23	**2 days**	0	7/10	0/10	5/10	0/10	*Cont×Ble: = 0.002
		1	3/10	0/10	4/10	3/10	*IRL×Ble-IRL: = 0.016
		2	0/10	0/10	1/10	7/10	*Ble×Ble-IRL: < 0.001
		3	0/10	6/10	0/10	0/10	Cont×IRL: = 0.335
		4	0/10	4/10	0/10	0/10	
		Median	0	3	0	2	
	30 days	0	7/10	0/10	6/10	0/10	*Cont×Ble: = 0.004
		1	3/10	4/10	4/10	6/10	*IRL×Ble-IRL: = 0.016
		2	0/10	4/10	0/10	4/10	Ble×Ble-IRL: = 0.257
		3	0/10	2/10	0/10	0/10	Cont×IRL: = 0.681
		4	0/10	0/10	0/10	0/10	
		Median	0	2	0	1	
HIF-1α	**2 days**	0	7/10	0/10	6/10	0/10	*Cont×Ble: = 0.002
		1	3/10	0/10	4/10	0/10	*IRL×Ble-IRL: = 0.002
		2	0/10	3/10	0/10	7/10	Ble×Ble-IRL: = 0.089
		3	0/10	7/10	0/10	3/10	Cont×IRL: = 0.681
		4	0/10	0/10	0/10	0/10	
		Median	0	3	0	2	
	30 days	0	8/10	0/10	5/10	0/10	*Cont×Ble: = 0.002
		1	2/10	0/10	5/10	0/10	*IRL×Ble-IRL: = 0.002
		2	0/10	5/10	0/10	2/10	Ble×Ble-IRL: = 0.185
		3	0/10	5/10	0/10	8/10	Cont×IRL: = 0.185
		4	0/10	0/10	0/10	0/10	
		Median	0	2	0	3	

Cont×Ble and IRL×Ble-IRL = Wilcoxon test ($P < 0.05$);
Ble×Ble-IRL and Cont×IRL = Mann-Whitney test ($P < 0.05$).
*The symbol highlights the significant difference among groups.

Discussion

This study evaluated *in vivo* the influence of LLLT applied after dental bleaching on the inflammatory process, immunolabeling of IL-23 cytokine and of the angiogenesis marker (HIF-1α). A significant reduction in the inflammatory infiltrate and in the presence of necrosis was observed in the group that received LLLT compared with the group that did not receive it. We also observed difference in immunolabeling of IL-23, with a reduction of severe immunolabeling in the Ble group to moderate immunolabeling in the Ble-IRL group. However, the immunolabeling of HIF-1α did not

differ significantly between the bleached groups, regardless of the application of LLLT. Thus, the null hypothesis that LLLT not influences the inflammation in the pulp of bleached teeth was rejected, but the null hypothesis that LLLT not influences the HIF-1α immunolabeling in pulp after bleaching was accepted.

A previous study compared the action of red laser (RL) and IRL on the pulp tissue of rat molars after bleaching, and observed similar effects in reducing the inflammatory infiltrate with a single application of IRL as well as with three applications of RL (Terayama *et al*. 2020, *in press*). Thus, the present study used only a single application of the IRL, due to reduction of inflammation on the dental pulp besides shorter clinical time. Other previous study which evaluated the potential of LLLT to minimize the damage caused by dental bleaching to pulp cells, showed that the higher reduction in the cytotoxicity of the bleaching gel was obtained using IRL compared to RL (Dantas *et al*. 2010), which supports our results.

Studies have shown that the beneficial effects of LLLT are directly related to its ability to stimulate cell proliferation (Martens *et al*. 2011, AlGhamdi *et al*. 2012, Basso *et al*. 2012), which was observed in pulp cells after stimulation (Yamakawa *et al*. 2018). Induction of gene expression of these cells and stimulation of dentin production was also observed (Yamakawa *et al*. 2018). In pulpotomy, LLLT stimulated the repair and promoted the healing of the pulp tissue (Saltzman *et al*. 2005, Fernandes *et al*. 2014, Marques *et al*. 2014), besides presenting anti-inflammatory potential (Olivi *et al*. 2009, Golpayegani *et al*. 2010, Martens *et al*. 2011). In addition, LLLT favoured the reduction of the exudative phase of the inflammatory process, and promoted an increase in vascularization and collagen synthesis (Olivi *et al*. 2009, Cannon *et al*. 2011, De Coster *et al*. 2013). These studies show the beneficial effects of LLLT for the repair of pulp tissue, which corroborates with the results observed in the present study.

Previously, an increased expression of pro-inflammatory cytokines was observed in the pulp tissue of bleached teeth (Lu *et al*. 2002, Silva *et al*. 2009, Ferreira *et al*. 2018, Benetti *et al*. 2018a, 2018b). The production of IL-23 cytokine is related to the formation of determined cell types, such as antigen-presenting cells, for example, monocytes, activated dendritic cells, and macrophages (Zhang *et al*. 2005, Kim *et al*. 2007, Boniface *et al*. 2008). It has been shown that IL-23 is important and necessary for the expansion and stabilization of cells in chronic inflammatory reactions (Harrington *et al*. 2005). Thus, the reduction in the immunolabeling of this cytokine would be a beneficial indicator of the effects of different therapies.

This was observed by previous study that evaluated the action of LLLT in the treatment of cutaneous inflammation, showing that with laser application the expression of IL-23 was suppressed (Rácz *et al*. 2010). The present study showed that the IRL was able to reduce significantly the immunolabeling of IL-23, being an indicator of the effectiveness of this therapy in reducing the damage caused by the bleaching gel to the pulp tissue.

Not only the expression of pro-inflammatory cytokines, but also the hypoxia is a factor directly related to tissue inflammation at the molecular level (Eltzschig *et al*. 2014). In these cases, the stabilization of HIF-1α is boosted, promoting cell adaptation to the hypoxic environment (Bakirtzi *et al*. 2014). HIF-1α is intended to promote angiogenesis to meet the need for oxygen, and it was found in endothelial cells, osteoblasts and inflammatory cells (Gomes-Filho *et al*. 2015). Moreover, it was activated during active inflammation by metabolic shifts toward hypoxia (Bakirtzi *et al*. 2014).

Hypoxia is a result of a manifestation of cellular stress, and is also related to the progressive growth of solid tumours cells, which acquire survival and proliferation capacity even in low oxygen tissues (Brown *et al*. 2004). It was demonstrated that LLLT could modulate HIF-1α, promoting a therapeutic approach by reduce hypoxia resulting in reduced expression of this marker in macrophages that coordinate the inflammatory process (Hsieh *et al*. 2012). However, a study that evaluated the influence of LLLT in immunolabeling of HIF-1α in cancerous tissue observed that an increased expression of this marker according to the increase in the number of applications of LLLT (Rhee *et al*. 2016). This may be a result of the induction of angiogenesis that LLLT can provide to the tissue (Abi-Ramia *et al*. 2010, Colombo *et al*. 2013).

We observed that the pulp tissue of bleached teeth had a higher immunolabeling for HIF-1α. It is known that cells exposed to hypoxia have higher levels of HIF-1α than normal cells (Cho *et al*. 2018), which corroborates with the results found in this study, since H_2O_2 promotes increased oxidative stress on the pulp tissue (Eimar *et al*. 2012, Briso *et al*. 2015, Cintra *et al*. 2016a, Benetti *et al*. 2017), as is found in a tissue in cancerous environment (Rhee *et al*. 2016).

However, the present data showed that LLLT was not able to significantly change the immunolabeling of HIF-1α after damages caused by H_2O_2 to the pulp tissue, despite of tending to a later increase in the immunolabeling of this marker. Previously,

it was observed that ROS act in the stabilization of HIF-1α (Xu *et al*. 2019), which may explain the results of this study.

Thus, considering that the effects of LLLT in various treatments are still questionable (Monteiro *et al*. 2011, Rhee *et al*. 2016, Benetti *et al*. 2018), further studies should be conducted to determine an ideal protocol, according to each clinical indication. Overall, through the data observed in this study and previous studies (Dantas *et al*. 2010, Terayama *et al*. 2020, *in press*), we can suggest that LLLT represents satisfactory results in minimizing inflammatory processes in pulp tissue. However, LLLT was not able to allow the recovery of a pulp tissue without changes, even 30 days after the bleaching, since there was a significant difference compared to its control group. Moreover, this study highlight that the H_2O_2 effects in pulp tissue are so intense that they can prevent the full action of therapies as the LLLT. This reinforces the importance of using low concentrations of H2O2 in the bleaching gel (The Council of The European Union 2011).

Conclusion

The LLLT, through the use of IRL, minimized the inflammatory infiltrate and IL-23 immunolabeling in the pulp tissue after dental bleaching, but did not influenced HIF-1α immunolabeling.

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III. Anexos

- Guidelines for Publishing Papers in the International Endodontic Journal
- Comitê de Ética do Uso de Animais

- Guidelines for Publishing Papers in the International Endodontic Journal

1. GENERAL

International Endodontic Journal publishes original scientific articles, reviews, clinical articles and case reports in the field of Endodontology; the branch of dental sciences dealing with health, injuries to and diseases of the pulp and periradicular region, and their relationship with systemic well-being and health. Original scientific articles are published in the areas of biomedical science, applied materials science, bioengineering, epidemiology and social science relevant to endodontic disease and its management, and to the restoration of root-treated teeth. In addition, review articles, reports of clinical cases, book reviews, summaries and abstracts of scientific meetings and news items are accepted.

Please read the instructions below carefully for details on the submission of manuscripts, the journal's requirements and standards as well as information concerning the procedure after a manuscript has been accepted for publication in International Endodontic Journal. Authors are encouraged to visit Wiley Blackwell Author Services for further information on the preparation and submission of articles and figures.

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Experimentation involving human subjects will only be published if such research has been conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki (version 2008) and the additional requirements, if any, of the country where the research has been carried out. Manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and written consent of each subject and according to the above mentioned principles. A statement regarding the fact that the study has been independently reviewed and approved by an ethical board should also be included.

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2.5 DNA Sequences and Crystallographic Structure Determinations

Papers reporting protein or DNA sequences and crystallographic structure determinations will not be accepted without a Genbank or Brookhaven accession number, respectively. Other supporting data sets must be made available on the publication date from the authors directly.

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Prior to acceptance there is no requirement to inform an Editorial Office that you intend to publish your paper Online Open if you do not wish to. All Online Open articles are treated in the same way as any other article. They go through the journal's standard peer-review process and will be accepted or rejected based on their own merit.

3.1 MANUSCRIPT SUBMISSION PROCEDURE

Manuscripts should be submitted electronically via the online submission site http://mc.manuscriptcentral.com/iej. The use of an online submission and peer review site enables immediate distribution of manuscripts and consequentially speeds up the review process. It also allows authors to track the status of their own manuscripts. Complete instructions for submitting a paper is available online and below. Further assistance can be obtained from iejeditor@cardiff.ac.uk.

3.2. Getting Started

• Launch your web browser (supported browsers include Internet Explorer 5.5 or higher, Safari 1.2.4, or Firefox 1.0.4 or higher) and go to the journal's online Submission Site: http://mc.manuscriptcentral.com/iej

• Log-in, or if you are a new user, click on 'register here'.

• If you are registering as a new user.

- After clicking on 'register here', enter your name and e-mail information and click 'Next'. Your e-mail information is very important.
- Enter your institution and address information as appropriate, and then click 'Next.'
- Enter a user ID and password of your choice (we recommend using your e-mail address as your user ID), and then select your areas of expertise. Click 'Finish'.

• If you are registered, but have forgotten your log in details, please enter your e-mail address under 'Password Help'. The system will send you an automatic user ID and a new temporary password.

• Log-in and select 'Author Centre '

3.3. Submitting Your Manuscript

• After you have logged into your 'Author Centre', submit your manuscript by clicking on the submission link under 'Author Resources'.

• Enter data and answer questions as appropriate. You may copy and paste directly from your manuscript and you may upload your pre-prepared covering letter.

• Click the 'Next' button on each screen to save your work and advance to the next screen.

• You are required to upload your files.

- Click on the 'Browse' button and locate the file on your computer.
- Select the designation of each file in the drop down next to the Browse button.
- When you have selected all files you wish to upload, click the 'Upload Files' button.

• Review your submission (in HTML and PDF format) before completing your submission by sending it to the Journal. Click the 'Submit' button when you are finished reviewing.

3.4. Manuscript Files Accepted

Manuscripts should be uploaded as Word (.doc) or Rich Text Format (.rft) files (not write-protected) plus separate figure files. GIF, JPEG, PICT or Bitmap files are acceptable for submission, but only high-resolution TIF or EPS files are suitable for printing. The files will be automatically converted to HTML and PDF on upload and will be used for the review process. The text file must contain the abstract, main text, references, tables, and figure legends, but no embedded figures or Title page. The Title page should be uploaded as a separate file. In the main text, please reference figures as for instance 'Figure 1', 'Figure 2' etc to match the tag name you choose for the individual figure files uploaded. Manuscripts should be formatted as described in the Author Guidelines below.

3.5. Blinded Review

Manuscript that do not conform to the general aims and scope of the journal will be returned immediately without review. All other manuscripts will be reviewed by experts in the field (generally two referees). International Endodontic Journal aims to forward referees´ comments and to inform the corresponding author of the result of the review process. Manuscripts will be considered for fast-track publication under special circumstances after consultation with the Editor. International Endodontic Journal uses double blinded review. The names of the reviewers will thus not be disclosed to the author submitting a paper and the name(s) of the author(s) will not be disclosed to the reviewers.

To allow double blinded review, please submit (upload) your main manuscript and title page as separate files. Please upload:

• Your manuscript without title page under the file designation 'main document'

• Figure files under the file designation 'figures'

• The title page and Acknowledgements where applicable, should be uploaded under the file designation 'title page'

All documents uploaded under the file designation 'title page' will not be viewable in the html and pdf format you are asked to review in the end of the submission process. The files viewable in the html and pdf format are the files available to the reviewer in the review process.

3.6. Suspension of Submission Mid-way in the Submission Process

You may suspend a submission at any phase before clicking the 'Submit' button and save it to submit later. The manuscript can then be located under 'Unsubmitted Manuscripts' and you can click on 'Continue Submission' to continue your submission when you choose to.

3.7. E-mail Confirmation of Submission

After submission you will receive an e-mail to confirm receipt of your manuscript. If you do not receive the confirmation e-mail after 24 hours, please check your e-mail address carefully in the system. If the e-mail address is correct please contact your IT department. The error may be caused by some sort of spam filtering on your email server. Also, the e-mails should be received if the IT department adds our e-mail server (uranus.scholarone.com) to their whitelist.

3.8. Manuscript Status

You can access ScholarOne Manuscripts any time to check your 'Author Centre' for the status of your manuscript. The Journal will inform you by e-mail once a decision has been made.

3.9. Submission of Revised Manuscripts

To submit a revised manuscript, locate your manuscript under 'Manuscripts with Decisions' and click on 'Submit a Revision'. Please remember to delete any old files uploaded when you upload your revised manuscript.

4. MANUSCRIPT TYPES ACCEPTED

Original Scientific Articles: must describe significant and original experimental observations and provide sufficient detail so that the observations can be critically evaluated and, if necessary, repeated. Original Scientific Articles must conform to the highest international standards in the field.

Review Articles: are accepted for their broad general interest; all are refereed by experts in the field who are asked to comment on issues such as timeliness, general interest and balanced treatment of controversies, as well as on scientific accuracy.

Reviews should generally include a clearly defined search strategy and take a broad view of the field rather than merely summarizing the authors´ own previous work.

Extensive or unbalanced citation of the authors´ own publications is discouraged. Mini Review Articles: are accepted to address current evidence on well-defined clinical, research or methodological topics. All are refereed by experts in the field who are asked to comment on timeliness, general interest, balanced treatment of controversies, and scientific rigor. A clear research question, search strategy and balanced synthesis of the evidence is expected. Manuscripts are limited in terms of word-length and number of figures.

Clinical Articles: are suited to describe significant improvements in clinical practice such as the report of a novel technique, a breakthrough in technology or practical approaches to recognised clinical challenges. They should conform to the highest scientific and clinical practice standards.

Case Reports: illustrating unusual and clinically relevant observations are acceptable but they must be of sufficiently high quality to be considered worthy of publication in the Journal. On rare occasions, completed cases displaying non-obvious solutions to significant clinical challenges will be considered. Illustrative material must be of the highest quality and healing outcomes, if appropriate, should be demonstrated.

Supporting Information: International Endodontic Journal encourages submission of adjuncts to printed papers via the supporting information website (see submission of supporting information below). It is encouraged that authors wishing to describe novel procedures or illustrate cases more fully with figures and/or video may wish to utilise this facility. Letters to the Editor: are also acceptable. Meeting Reports: are also acceptable.

5. MANUSCRIPT FORMAT AND STRUCTURE

5.1. Format

Language: The language of publication is English. It is preferred that manuscript is professionally edited. A list of independent suppliers of editing services can be found at http://authorservices.wiley.com/bauthor/english_language.asp. All services are paid for and arranged by the author, and use of one of these services does not guarantee acceptance or preference for publication.

Presentation: Authors should pay special attention to the presentation of their research findings or clinical reports so that they may be communicated clearly.

Technical jargon should be avoided as much as possible and clearly explained where its use is unavoidable. Abbreviations should also be kept to a minimum, particularly those that are not standard. The background and hypotheses underlying the study, as well as its main conclusions, should be clearly explained. Titles and abstracts especially should be written in language that will be readily intelligible to any scientist.

Abbreviations: International Endodontic Journal adheres to the conventions outlined in Units, Symbols and Abbreviations: A Guide for Medical and Scientific Editors and Authors. When non-standard terms appearing 3 or more times in the manuscript are to be abbreviated, they should be written out completely in the text when first used with the abbreviation in parenthesis.

5.2. Structure

All manuscripts submitted to International Endodontic Journal should include Title Page, Abstract, Main Text, References and Acknowledgements, Tables, Figures and Figure Legends as appropriate Title Page: The title page should bear: (i) Title, which should be concise as well as descriptive; (ii) Initial(s) and last (family) name of each author; (iii) Name and address of department, hospital or institution to which work should be attributed; (iv) Running title (no more than 30 letters and spaces); (v) No more than six keywords (in alphabetical order); (vi) Name, full postal address, telephone, fax number and e-mail address of author responsible for correspondence.

Abstract for Original Scientific Articles should be no more than 250 words giving details of what was done using the following structure:

• Aim: Give a clear statement of the main aim of the study and the main hypothesis tested, if any.

• Methodology: Describe the methods adopted including, as appropriate, the design of the study, the setting, entry requirements for subjects, use of materials, outcome measures and statistical tests.

• Results: Give the main results of the study, including the outcome of any statistical analysis.

• Conclusions: State the primary conclusions of the study and their implications.

Suggest areas for further research, if appropriate.

Abstract for Review Articles should be non-structured of no more than 250 wordsgiving details of what was done including the literature search strategy.

Abstract for Mini Review Articles should be non-structured of no more than 250words, including a clear research question, details of the literature search strategyand clear conclusions.

Abstract for Case Reports should be no more than 250 words using the following structure:

• Aim: Give a clear statement of the main aim of the report and the clinical problem which is addressed.

• Summary: Describe the methods adopted including, as appropriate, the design of the study, the setting, entry requirements for subjects, use of materials, outcome measures and analysis if any.

• Key learning points: Provide up to 5 short, bullet-pointed statements to highlight the key messages of the report. All points must be fully justified by material presented in the report.

Abstract for Clinical Articles should be no more than 250 words using the following structure:

• Aim: Give a clear statement of the main aim of the report and the clinical problem which is addressed.

• Methodology: Describe the methods adopted.

• Results: Give the main results of the study.

• Conclusions: State the primary conclusions of the study.

Main Text of Original Scientific Article should include Introduction, Materials and Methods, Results, Discussion and Conclusion Introduction: should be focused, outlining the historical or logical origins of the study and gaps in knowledge. Exhaustive

literature reviews are not appropriate. It should close with the explicit statement of the specific aims of the investigation, or hypothesis to be tested.

Material and Methods: must contain sufficient detail such that, in combination with the references cited, all clinical trials and experiments reported can be fully reproduced.

(i) Clinical Trials should be reported using the CONSORT guidelines available at www.consort-statement.org. A CONSORT checklist and flow diagram (as a Figure) should also be included in the submission material.

(ii) Experimental Subjects: experimentation involving human subjects will only be published if such research has been conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki (version 2008) and the additional requirements, if any, of the country where the research has been carried out. Manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and written consent of each subject and according to the above mentioned principles. A statement regarding the fact that the study has been independently reviewed and approved by an ethical board should also be included. Editors reserve the right to reject papers if there are doubts as to whether appropriate procedures have been used.

When experimental animals are used the methods section must clearly indicate that adequate measures were taken to minimize pain or discomfort. Experiments should be carried out in accordance with the Guidelines laid down by the National Institute of Health (NIH) in the USA regarding the care and use of animals for experimental procedures or with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in accordance with local laws and regulations.

All studies using human or animal subjects should include an explicit statement in the Material and Methods section identifying the review and ethics committee approval for each study, if applicable. Editors reserve the right to reject papers if there is doubt as to whether appropriate procedures have been used.

(iii) Suppliers: Suppliers of materials should be named and their location (Company, town/city, state, country) included.

Results: should present the observations with minimal reference to earlier literature or to possible interpretations. Data should not be duplicated in Tables and Figures.

Discussion: may usefully start with a brief summary of the major findings, but repetition of parts of the abstract or of the results section should be avoided. The Discussion section should progress with a review of the methodology before discussing the results in light of previous work in the field. The Discussion should end with a brief conclusion and a comment on the potential clinical relevance of the findings. Statements and interpretation of the data should be appropriately supported by original references.

Conclusion: should contain a summary of the findings.

Main Text of Review Articles should be divided into Introduction, Review and Conclusions. The Introduction section should be focused to place the subject matter in context and to justify the need for the review. The Review section should be divided into logical sub-sections in order to improve readability and enhance understanding. Search strategies must be described and the use of state-of-the-art evidence-based systematic approaches is expected. The use of tabulated and illustrative material is encouraged. The Conclusion section should reach clear conclusions and/or recommendations on the basis of the evidence presented. Main Text of Mini Review Articles should be divided into Introduction, Review and Conclusions. The Introduction section should briefly introduce the subject matter and justify the need and timeliness of the literature review. The Review section should be divided into logical sub-sections to enhance readability and understanding and may be supported by up to 5 tables and figures. Search strategies must be described and the use of state-of-the-art evidence-based systematic approaches is expected. The Conclusions section should present clear statements/recommendations and suggestions for further work. The manuscript, including references and figure legends should not normally exceed 4000 words. Main Text of Clinical Reports and Clinical Articles should be divided into Introduction, Report, Discussion and Conclusion. They should be well illustrated with clinical images, radiographs, diagrams and, where appropriate, supporting tables and graphs. However, all illustrations must be of the highest quality Acknowledgements: International Endodontic Journal requires that all sources of institutional, private and corporate financial support for the work within the manuscript must be fully acknowledged, and any potential conflicts of interest noted. Grant or contribution numbers may be acknowledged, and principal grant holders should be listed. Acknowledgments should be brief and should not include thanks to anonymous referees and editors. See also above under Ethical Guidelines.

5.3. References

It is the policy of the Journal to encourage reference to the original papers rather than to literature reviews. Authors should therefore keep citations of reviews to the absolute minimum.

We recommend the use of a tool such as EndNote or Reference Manager for reference management and formatting. The EndNote reference style can be obtained upon request to the editorial office (iejeditor@cardiff.ac.uk). Reference Manager reference styles can be searched for here: www.refman.com/support/rmstyles.aspIn the text: single or double authors should be acknowledged together with the year of publication,

e.g. (Pitt Ford & Roberts 1990). If more than two authors the first author followed by *et al.* is sufficient, e.g. (Tobias *et al.* 1991). If more than 1 paper is cited the references should be in year order and separated by "," e.g. (Pitt Ford & Roberts 1990, Tobias *et al.* 1991).

Reference list: All references should be brought together at the end of the paper in alphabetical order and should be in the following form.

(i) Names and initials of up to six authors. When there are seven or more, list the first three and add *et al.*

(ii)Year of publication in parentheses

(iii) Full title of paper followed by a full stop (.)

(iv) Title of journal in full (in italics)

(v) Volume number (bold) followed by a comma (,)

(vi) First and last pages

Examples of correct forms of reference follow:

Standard journal article

Bergenholtz G, Nagaoka S, Jontell M (1991) Class II antigen-expressing cells in experimentally induced pulpitis. *International Endodontic Journal* **24**, 8-14.

Corporate author

British Endodontic Society (1983) Guidelines for root canal treatment. *International Endodontic Journal* **16**, 192-5.

Journal supplement

Frumin AM, Nussbaum J, Esposito M (1979) Functional asplenia: demonstration of splenic activity by bone marrow scan (Abstract). *Blood* **54** (Suppl. 1), 26a.

Books and other monographs

Personal author(s)

Gutmann J, Harrison JW (1991) Surgical Endodontics, 1st edn Boston, MA, USA: Blackwell Scientific Publications.

Chapter in a book Wesselink P (1990) Conventional root-canal therapy III: root filling. In: Harty FJ, ed. Endodontics in Clinical Practice, 3rd edn; pp. 186-223. London, UK: Butterworth.

Published proceedings paper

DuPont B (1974) Bone marrow transplantation in severe combined immunodeficiency with an unrelated MLC compatible donor. In: White HJ, Smith R, eds. Proceedings of the Third Annual Meeting of the International Society for Experimental Rematology; pp. 44-46. Houston, TX, USA: International Society for Experimental Hematology.

Agency publication Ranofsky AL (1978) Surgical Operations in Short-Stay Hospitals: United States- 1975. DHEW publication no. (PHS) 78-1785 (Vital and Health Statistics; Series 13; no. 34.) Hyattsville, MD, USA: National Centre for Health Statistics.8

Dissertation or thesis

Saunders EM (1988) In vitro and in vivo investigations into root-canal obturation using thermally softened gutta-percha techniques (PhD Thesis). Dundee, UK: University of Dundee.

URLs

Full reference details must be given along with the URL, i.e. authorship, year, title of document/report and URL. If this information is not available, the reference should be removed and only the web address cited in the text.

Smith A (1999) Select committee report into social care in the community [WWW document]. URL http://www.dhss.gov.uk/reports/report015285.html [accessed on 7 November 2003]

5.4. Tables, Figures and Figure Legends

Tables: Tables should be double-spaced with no vertical rulings, with a single bold ruling beneath the column titles. Units of measurements must be included in the column title.

Figures: All figures should be planned to fit within either 1 column width (8.0 cm), 1.5column widths (13.0 cm) or 2 column widths (17.0 cm), and must be suitable for photocopy reproduction from the printed version of the manuscript. Lettering on figures should be in a clear, sans serif typeface (e.g. Helvetica); if possible, the same type face should be used for all figures in a paper. After reduction for publication, upper-case text and numbers should be at least 1.5-2.0 mm high (10 point Helvetica). After reduction, symbols should be at least 2.0-3.0 mm high (10 point). All half-tone photographs should be submitted at final reproduction size. In general, multi-part figures should be arranged as they would appear in the final version. Reduction to the scale that will be used on the page is not necessary, but any special requirements (such as the separation distance of stereo pairs) should be clearly specified.

Unnecessary figures and parts (panels) of figures should be avoided: data presented in small tables or histograms, for instance, can generally be stated briefly in the text instead. Figures should not contain more than one panel unless the parts are logically connected; each panel of a multipart figure should be sized so that the whole figure can be reduced by the same amount and reproduced on the printed page at the smallest size at which essential details are visible.

Figures should be on a white background, and should avoid excessive boxing, unnecessary colour, shading and/or decorative effects (e.g. 3-dimensionalskyscraper histograms) and highly pixelated computer drawings. The vertical axis of histograms should not be truncated to exaggerate small differences. The line spacing should be wide enough to remain clear on reduction to the minimum acceptable printed size.

Figures divided into parts should be labelled with a lower-case, boldface, roman letter, a, b, and so on, in the same type size as used elsewhere in the figure. Lettering in figures should be in lower-case type, with the first letter capitalized. Units should have a single space between the number and the unit, and follow SI nomenclature or the nomenclature common to a particular field. Thousands should be separated by a thin space (1 000). Unusual units or abbreviations should be spelled out in full or defined in the legend. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. In general, visual cues (on the figures themselves) are preferred to verbal explanations in the legend (e.g. broken line, open red triangles etc.). Figure legends: Figure legends should begin with a brief title for the whole figure and continue with a short description of each panel and the symbols used; they should not contain any details of methods.

Permissions: If all or part of previously published illustrations are to be used, permission must be obtained from the copyright holder concerned. This is the responsibilty of the authors before submission.

Preparation of Electronic Figures for Publication: Although low quality images are adequate for review purposes, print publication requires high quality images to prevent the final product being blurred or fuzzy. Submit EPS (line art) or TIFF(halftone/photographs) files only. MS PowerPoint and Word Graphics are unsuitable for printed pictures. Do not use pixel-oriented programmes. Scans (TIFF only) should have a resolution of 300 dpi (halftone) or 600 to 1200 dpi (line drawings) in relation to the reproduction size (see below). EPS files should be saved with fonts embedded (and with a TIFF preview if possible). For scanned images, the scanning resolution (at final image size) should be as follows to ensure good reproduction: line art: >600dpi; half-tones (including gel photographs): >300 dpi; figures containing both halftone and line images: >600 dpi.

Further information can be obtained at Wiley Blackwell’s guidelines for figures: http:/authorservices.wiley.com/bauthor/illustration.asp.

Check your electronic artwork before submitting it: http://authorservices.wiley.com/bauthor/eachecklist.asp.

5.5. Supporting Information

Publication in electronic formats has created opportunities for adding details or whole sections in the electronic version only. Authors need to work closely with the editors in developing or using such new publication formats.

Supporting information, such as data sets or additional figures or tables, that will not be published in the print edition of the journal, but which will be viewable via the online edition, can be submitted. It should be clearly stated at the time of submission that the supporting information is intended to be made available through the online edition. If the size or format of the supporting information is such that it cannot be accommodated on the journal's website, the author agrees to make the supporting information available free of charge on a permanent Web site, to which links will be set up from the journal's website. The author must advise Wiley Blackwell if the URL of the website where the supporting information is located changes. The content of the supporting information must not be altered after the paper has been accepted for publication.

The availability of supporting information should be indicated in the main manuscript by a paragraph, to appear after the References, headed 'Supporting Information' and providing titles of figures, tables, etc. In order to protect reviewer anonymity, material posted on the authors Web site cannot be reviewed. The supporting information is an integral part of the article and will be reviewed accordingly.

Preparation of Supporting Information: Although provision of content through the web in any format is straightforward, supporting information is best provided either in web ready form or in a form that can be conveniently converted into one of the standard web publishing formats:

• Simple word-processing files (.doc or .rtf) for text.

• PDF for more complex, layout-dependent text or page-based material. Acrobat files can be distilled from Postscript by the Publisher, if necessary.

• GIF or JPEG for still graphics. Graphics supplied as EPS or TIFF a real so acceptable.

• MPEG or AVI for moving graphics.

Subsequent requests for changes are generally unacceptable, as for printed papers.

A charge may be levied for this service. Video Imaging: For the on-line version of the Journal the submission of illustrative video is encouraged. Authors proposing the use such media should consult with the Editor during manuscript preparation.

- Comitê de Ética do Uso de Animais

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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 **"Influência da laserterapia de baixa intensidade no processo inflamatório e na capacidade reparadora do tecido pulpar de dentes clareados"**, Processo FOA nº 00713-2018, 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 13 de Novembro de 2018.

VALIDADE DESTE CERTIFICADO: 31 de Novembro de 2020.
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 31 de Dezembro de 2020.

CERTIFICATE

We certify that the study entitled **"Influence of low intensity laser therapy on the inflammatory process and on the repair capacity of the pulp tissue of bleached teeth"**, Protocol FOA nº 00713-2018, 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 November 13, 2018.

VALIDITY OF THIS CERTIFICATE: November 31, 2020.
DATE OF SUBMISSION OF THE FINAL REPORT: December 31, 2020.

Profa. Associada Maria Cristina Rosifini Alves Rezende
Coordenador da CEUA
CEUA Coordinator

CEUA - Comissão de Ética no Uso de Animais
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