Juliana Goto

INFLUÊNCIA DA DIABETES MELLITUS NO PROCESSO INFLAMATÓRIO, REGENERATIVO E NA DEGRADAÇÃO DE MATRIZ EXTRACELULAR DO TECIDO PULPAR DE RATOS SUBMETIDOS A CLAREAÇÃO

Araçatuba – SP
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Orientador: Prof. Assoc. Luciano Tavares Angelo Cintra

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Dedico este trabalho à minha mãe, a pessoa mais resiliente e forte que conheço. Sua coragem me inspirou, seu suporte me manteve determinada e seu amor me trouxe até aqui.

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“Se vi mais longe, foi por estar sobre ombros de gigantes.”

Isaac Newton.

GOTO, J. **Influência da diabetes mellitus no processo inflamatório, regenerativo e na degradação de matriz extracelular do tecido pulpar de ratos submetidos a clareação.** 2023. 77 f. Dissertação (Mestrado) - Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba, 2023.

RESUMO GERAL

Introdução: O procedimento de clareação dentária com a utilização do peróxido de hidrogênio (H2O2) está associada a danos ao tecido pulpar, que variam de inflamação leve à necrose. Estudos demonstraram que a presença de diabetes mellitus (Dm) pode influenciar em alguns parâmetros avaliados. **Objetivos:** avaliar *in vivo* a influência da diabetes mellitus (Dm) no processo inflamatório, regenerativo e na degradação da matriz extracelular do tecido pulpar de ratos após clareação dentária, analisando a interleucina (IL) 6 e (IL)10, os fatores de crescimento transformante (TGF)-β e de fibroblastos (FGF)-2, marcadores de metaloproteinases MMP-2 e MMP-9 e inibidores de metaloproteinases TIMP-1 e TIMP-2. **Métodos:** Setenta ratos Wistar foram utilizados, sendo metade normoglicêmicos (N) e metade diabéticos (D), induzidos por estreptozotocina. A clareação dentária (H2O2 a 17,5% por 30 min) foi realizada nos molares superiores formando os grupos: N, D, NCla (normoglicêmico clareado) e DCla (diabético clareado). Após 0h, 2, 7, 15 e 30 dias (n=7), os animais foram eutanasiados e as maxilas removidas para avaliação histológica em H.E. e imunoistoquímica via densidade óptica de imunomarcação (DoI). Testes estatísticos de Wilcoxon e Mann-Whitney foram aplicados ($p<0,05$). **Resultados:** Em 0h, NCla e DCla apresentaram necrose pulpar. Já nos períodos de 2 e 7 dias o grupo DCla apresentou inflamação mais intensa e maior DoI para IL-6 que NCla ($p<0,05$). Aos 15 dias, NCla apresentou menor DoI para IL-6 e IL-10 que DCla ($p<0,05$). Para TGF-β, aos 2 dias NCla apresentou maior DoI que DCla ($p<0,05$). Para FGF-2 em 0h e 2 dias, NCla apresentou DoI maior que DCla ($p<0,05$). Esta diferença desapareceu aos 7 dias ($p>0,05$) e se inverteu aos 15 dias ($p<0,05$). Os grupos NCla e DCla apresentaram semelhança na DoI para MMP-2, MMP-9 e TIMP-1 nos períodos de 0h, 2, 7 e 15 dias ($p>0,05$). Já no período de 30 dias o grupo DCla apresentou maior DoI para MMP-2, MMP-9 e TIMP-1 comparado a NCla ($p<0,05$). Em relação à TIMP-2, não foram encontradas diferenças entre os grupos ($p>0,05$). **Conclusão:** Conclui-se que a diabetes influencia na severidade da inflamação do tecido pulpar após clareação dentária, elevando a produção de IL-6 e

mantendo por maior período a produção de IL-10, assim como influencia no processo regenerativo, reduzindo a produção de TGF-β e retardando a produção de FGF-2. Ainda, influencia na degradação da matriz extracelular, mantendo elevada por maior período a produção de MMP-2 e MMP-9, assim como de TIMP-1.

Palavras-chave: Clareação dentária. Diabetes mellitus. Interleucinas. Metaloproteinases da matriz. Inibidores teciduais das metaloproteinases.

GOTO, J. **Influence of diabetes mellitus on inflammation, regeneration and extracellular matrix degradation in the pulp tissue of rats submitted to bleaching.** 2023. 77 f. Dissertação (Mestrado) - Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba, 2023.

GENERAL ABSTRACT

Introduction: The dental bleaching procedure using hydrogen peroxide (H2O2) is associated with pulp tissue damage, ranging from mild inflammation to necrosis. Studies have shown that the presence of diabetes mellitus (Dm) can influence some parameters evaluated. **Objectives:** To evaluate in vivo the influence of diabetes mellitus (DM) on inflammation, regeneration and extracellular matrix degradation in rat pulp tissue after dental bleaching by analyzing interleukin (IL) 6 and (IL)10, transforming growth factor (TGF)-β and fibroblast growth factor (FGF)-2, metalloproteinases markers MMP-2 and MMP-9, and metalloproteinases inhibitors TIMP-1 and TIMP-2. **Methods:** Seventy Wistar rats were used, half normoglycemic (N) and half diabetic (D), induced by streptozotocin. Dental bleaching (H2O2 17.5% for 30 min) was performed on the upper molars forming the groups: N, D, NBle (bleached normoglycemic) and DBle (bleached diabetic). After 0h, 2, 7, 15 and 30 days (n=7), the animals were euthanized and the maxillae removed for histological evaluation in H.E. and immunohistochemistry via optical density immunolabeling (ODI). Wilcoxon and Mann-Whitney statistical tests were applied ($p<0.05$). **Results:** At 0h, NBle and DBle showed pulp necrosis. At 2 and 7 days, the DBle group showed more intense inflammation and higher ODI for IL-6 than NBle ($p<0.05$). At 15 days, NBle showed lower ODI for IL-6 and IL-10 than DBle ($p<0.05$). For TGF-β, at 2 days NBle showed higher ODI than DBle ($p<0.05$). For FGF-2 at 0h and 2 days, NBle showed higher ODI than DBle ($p<0.05$). This difference disappeared at 7 days ($p>0.05$) and reversed at 15 days ($p<0.05$). The NBle and DBle groups showed similarity in the ODI for MMP-2, MMP-9 and TIMP-1 at 0h, 2, 7 and 15 days ($p>0.05$). At 30 days, the DBle group presented higher ODI for MMP-2, MMP-9 and TIMP-1 compared to the NBle group ($p<0.05$). Regarding TIMP-2, no differences were found between groups ($p>0.05$). **Conclusion:** It was concluded that diabetes influences the severity of pulp tissue inflammation after dental bleaching, increasing the production of IL-6 and maintaining the production of IL-10 for a longer period, as well as influencing the regenerative process, reducing the production of TGF-β and delaying

the production of FGF-2. It also influences the degradation of the extracellular matrix, keeping the production of MMP-2 and MMP-9, as well as TIMP-1, elevated for a longer period.

Keywords: Dental whitening. Diabetes mellitus. Interleukins. Matrix metalloproteinases. Tissue inhibitors of metalloproteinases.

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LISTA DE ABREVIATURAS

Capes	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
D	diabetic
DBle	bleached diabetic
Dm	diabetes mellitus
ECM	extracellular matrix
EDTA	ácido etilenodiamino tetra-acético
EROs	espécies reativas de oxigênio
FGF-2	fator de crescimento de fibroblastos
Fig.	figure
G	grama
H	horas
H.E	hematoxilina-eosina
H_2O_2	peróxido de hidrogênio
IL-10	interleucina 10
IL-17	interleucina 17
IL-6	interleucina 6
Kg	quilograma
Mg dl^{-1}	miligramas de glicose por decilitro de sangue
Mg	miligrama
MMP-2	metaloproteinase de matriz 2
MMP-9	metaloproteinase de matriz 9
MMPs	metaloproteinases de matriz
N	normoglycemic
NBle	bleached normoglycemic
ODI	optical density immunostaining
PBS	phosphate-buffered saline
ROS	reactive oxygen species
SD	standard deviation
TGF-β	fator de crescimento transformante beta
TIMP-1	inibidor tecidual das metaloproteinases 1
TIMP-2	inibidor tecidual das metaloproteinases 2

TIMPs	inibidores teciduais das metaloproteinases
TNF-α	fator de necrose tumoral alfa

SUMÁRIO

1 INTRODUÇÃO GERAL *

A procura por uma estética dentária considerada satisfatória, de acordo com os padrões de beleza da sociedade moderna, tornou a clareação dentária um dos procedimentos mais populares nos consultórios odontológicos (AGOSTINHO; GUIMARÃES; SILVA, 2003, ALMEIDA *et al.,* 2015). A insatisfação dos pacientes relacionada, principalmente, com a coloração escurecida dos dentes (TIN-OO; SADDKI; HASSAN, 2011), bem como, a manipulação acessível dos produtos, favorecem a utilização de géis clareadores (AGOSTINHO; GUIMARÃES; SILVA, 2003). Um dos componentes dos agentes clareadores que tem sido amplamente utilizado é o peróxido de hidrogênio (H_2O_2), um ativo capaz de se dissociar em espécies reativas de oxigênio (EROs) que permeiam por esmalte e dentina permitindo a eficácia clareadora (BENETTI *et al.,* 2004; CAMPS *et al.,* 2007, 2010; CARRASCO-GUERISOLI *et al.,* 2009; CINTRA *et al.,* 2016; HANNIG *et al.,* 2011).

No entanto, foi demonstrado que o procedimento clareador está associado à alterações dos tecidos dentários, atingindo o tecido pulpar (CINTRA *et al.* 2017; FERREIRA *et al.,* 2018). Foi observado que ao entrar em contato com o tecido pulpar, as EROs provocam um processo inflamatório que pode variar de discreto à severo, redução da celularidade e do metabolismo celular (BENETTI *et al*., 2018b; CINTRA *et al.,* 2013; MIN *et al.,* 2008; SEALE; MCINTOSH; TAYLOR, 1981; SOARES *et al.,* 2014).

Sabe-se que alterações sistêmicas podem prejudicar de forma direta os tecidos dentários (JUNG *et al*., 2013; LIMA *et al*., 2013). A diabetes mellitus (Dm) é uma síndrome de alcance mundial, responsável por alterações na metabolização de lipídeos, carboidratos e proteínas devido a hiperglicemia (LIMA *et al*., 2013; SUN *et al*., 2011). Em estudos anteriores foi observado que a diabetes mellitus (Dm) possui relação estabelecida com alterações endodônticas (CINTRA *et al*., 2014a, 2014b, 2017, FERREIRA *et al*., 2017, PRIETO *et al*., 2017). Desse modo, pacientes diabéticos apresentam maior susceptibilidade a complicações orais (BENDER; BENDER, 2003, FOUAD; BURLESON, 2003), tendo como consequência uma polpa dentária diretamente afetada (CATANZARO *et al.,* 2006; INAGAKI *et al*., 2010; LEITE *et al*., 2008, 2010), inúmeras alterações como redução do colágeno

* Referências no Anexo A

(BENDER; BENDER, 2003, LEITE *et al.,* 2008), e circulação sanguínea prejudicada, resultando em aumento no risco de necrose (BENDER; BENDER, 2003, CATANZARO *et al.,* 2006). Em outros trabalhos também foi demonstrado que a DM possui influência nas alterações da produção de algumas citocinas pró-inflamatórias em diversas doenças orais (AMIR *et al.,* 2011; SUN *et al*., 2011),

Algumas citocinas são importantes indicadores de modificação do processo inflamatório (FARGES *et al.,* 2015). A interleucina (IL)-6 é uma citocina pleotrópica e pró-inflamatória (FERREIRA *et al*., 2018), produzida pela ação sinérgica do TNF-α (SCHINDLER *et al.,* 1990) e (IL)-17 (XIONG; PENG, 2015). A IL-6 é capaz de promover a atração de neutrófilos e o aumento da permeabilidade vascular na câmara pulpar (ELSALHY; AZIZIEH; RAGHUPATHY, 2013, XIONG; PENG, 2015) aumentando o edema (ELSALHY; AZIZIEH; RAGHUPATHY, 2013) e contribuindo para a inflamação deste tecido (AZUMA *et al.,* 2015). Essa citocina foi observada em células pulpares humanas após clareação dentária (SOARES *et al.,* 2015) e, no processo inflamatório que ocorre no tecido pulpar de ratos após o procedimento clareador, principalmente nos períodos iniciais (BENETTI *et al.,* 2018a).

Células imunes e não imunes que modulam a resposta inflamatória são capazes de produzir citocinas, sendo uma delas a interleucina (IL)-10, uma citocina imunossupressora que atua impedindo que inflamações se tornem excessivas ou desnecessárias (FARGES *et al*., 2015). A IL-10 possui ação sobre algumas citocinas reduzindo sua expressão, tais como a IL-6 e TNF-α (LI; FLAVELL, 2008; MOREIRA *et al.,* 2009), já identificadas em tecido pulpar de dentes clareados (BENETTI *et al*., 2018a, FERREIRA *et al.,* 2018). Ademais, seu papel foi observado quando expressa em células odontoblastóides in vitro, o que sugeriu a capacidade dos odontoblastos de limitar a intensidade das respostas imune e inflamatória pulpar e não apenas de iniciá-las (FARGES *et al.,* 2015). Em estudos do nosso grupo, foi verificado que as células odontoblastóides reagem a agressores do complexo dentinho-pulpar, como o H_2O_2, compondo a primeira camada de células com expressão de citocinas (BENETTI *et al.,* 2018a).

Assim como a IL-10, o (TGF)-β também é um importante regulador com potentes efeitos imunossupressores (MACIEL *et al.,* 2012). Foi observado que o fator de crescimento é capaz de inibir a produção de citocinas pró-inflamatórias e

antagonizar atividades biológicas de algumas citocinas (COLIC *et al*., 2009). Estudos também demonstraram sua atuação na regulação de respostas pró-inflamatórias durante as fases agudas do desenvolvimento da lesão periapical (MACIEL *et al*., 2012).

Em conjunto com o (TGF)-β, o fator de crescimento de fibroblastos (FGF)-2 também apresentou atuação no controle da proliferação celular e apoptose, ambos eventos regulados e reativados durante o processo de reparação tecidual (WIDBILLER *et al*., 2018). Estudos observaram que o fator de crescimento de fibroblastos é capaz de promover a diferenciação *in* vitro de células pulpares em odontoblastos (HAO; WANG; SHI, 1999), assim como atuar no processo de angiogênese e desempenhar papel mitógeno para células progenitoras da polpa (ABOUT *et al*., 2000; MATHIEU *et al*., 2013; UNDA *et al*., 2001).

Algumas enzimas proteolíticas também atuam no reparo e na renovação tecidual por meio da regulação da matriz extracelular, dentre elas, as metaloproteinases de matriz (MMPs) (AVADANEI *et al*., 2013; CHANG *et al*., 2017; COGNI *et al*., 2013; KIM *et al*., 2012). Estudos demonstraram que na presença do H_2O_2 foi observado o aumento na degradação de colágeno mediada por MMPs no tecido dentinário, indicando a contribuição das enzimas para a remodelação do tecido pulpar (TOLEDANO *et al*., 2001). Outros trabalhos relataram a participação de MMP-2 e MMP-9 na destruição do tecido pulpar (GUSMAN; SANTANA; ZEHNDER, 2002; TSAI *et al*., 2005), bem como níveis significantes da MMP-9 em polpas inflamadas quando comparadas as polpas saudáveis (GUSMAN; SANTANA; ZEHNDER, 2002).

Juntamente com as MMPs, os inibidores teciduais das metaloproteinases (TIMPs) são responsáveis por regular em parte a atuação das MMPs, sendo secretados juntamente com essas (RODERFELD *et al*., 2007). Foi observado que TIMP-1 atua como o inibidor específico de MMP-9 (KIM *et al*., 2012) e TIMP-2 possui capacidade de atuar como modulador de MMP-2 (AVADANEI *et al*., 2013; KIM *et al*., 2012). O equilíbrio entre MMPs e TIMPs podem indicar a regulação da matriz extracelular do tecido pulpar danificado (WISITHPHROM; MURRAY; WINDSOR, 2006). No entanto, a ação das MMPs e seus inibidores não foi investigada no tecido pulpar de indivíduos diabéticos.

Assim, nesse estudo objetivou-se o melhor entendimento acerca da influência da diabetes mellitus sobre a inflamação do tecido pulpar, bem como o processo regenerativo e a degradação da matriz extracelular em espécimes submetidos à clareação dentária.

2 ARTIGO 1 - INFLUENCE OF DIABETES ON THE INFLAMMATORY AND REGENERATIVE PROCESS OF THE PULP TISSUE OF RAT TEETH SUBMITTED TO DENTAL BLEACHING†

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2.1 Abstract

Aim: To evaluate the influence of diabetes mellitus (Dm) on the inflammatory and regenerative process of the pulp tissue of rats after dental bleaching by analyzing interleukin (IL) 6 and 10 and transforming growth factors (TGF)-β and fibroblasts (FGF)-2.

Methodology: Seventy Wistar rats were divided into 2 groups (n = 35): normoglycemic (N) and diabetic (D). Dm was induced by streptozotocin and confirmed after 7 days. Then, dental bleaching with 17.5% H_2O_2 for 30 min was performed on the upper molars, forming the groups: N, D, NBle (bleached normoglycemic) and DBle (bleached diabetic). After 0-hour, 2, 7, 15 and 30 days, the animals were euthanized and the jaws removed and processed for histological evaluation in H.E. stain and immunohistochemistry for IL-6, IL-10, TGF-β and FGF-2 analysed via optical density immunostaining (ODI). Wilcoxon and Mann-Whitney statistical tests were applied ($p<0.05$).

† Normalizado de acordo com a Revista "International Endodontic Journal" - https://onlinelibrary.wiley.com/page/journal/13652591/homepage/forauthors.html?1

Results: At 0-hour, NBle and DBle showed pulp necrosis. In the periods of 2 and 7 days, the DBle group presented more intense inflammation and higher ODI for IL-6 than NBle ($p<0.05$). At 15 days, NBle showed lower ODI for IL-6 and IL-10 than DBle ($p<0.05$). For TGF-β, at 2 days NBle showed higher ODI than DBle ($p<0.05$). For FGF-2 at 0-hour and 2 days, NBle presented higher ODI than DBle ($p<0.05$). This difference disappeared at 7 days ($p>0.05$) and reversed at 15 days ($p<0.05$).

Conclusions: It is concluded that diabetes influences the severity of pulp tissue inflammation after dental bleaching, increasing the production of IL-6 and maintaining the production of IL-10 for a longer period, as well as influencing the regenerative process, reducing the production of TGF- β and delaying the production of FGF-2.

Keywords: dental bleaching; diabetes mellitus; inflammatory response; regenerative process; proinflammatory cytokines

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

2.2 Introduction

Dental bleaching occurs through the dissociation of H_2O_2 into reactive oxygen species (ROS) that can permeate enamel and dentin due to their low molecular weight (Benetti et al., 2004; Camps et al., 2007, 2010; Carrasco-Guerisoli et al., 2009; Cintra et al., 2016a; Hannig et al., 2011). It is known that when the bleaching gel reaches the dental pulp, it reacts with the cells and the extracellular matrix of the tissue, generating an inflammatory infiltrate (Cintra et al., 2013, 2016a, 2016b), alteration in the expression of inflammatory cytokines (Benetti et al., 2018b; Ferreira et al., 2018) and increase vascular permeability (Ferreira et al., 2013). During this reaction, tissue necrosis was also found in more severe cases (Benetti et al., 2017, 2018b ; Cintra et al., 2013, 2016b; Costa et al., 2010).

Previous studies have shown that hyperglycemic condition determines higher pulpal damage after bleaching procedures (Cintra et al., 2017a). Diabetes mellitus is a metabolic disorder whose systemic alteration can directly affect dental tissues (Lima et al., 2013; Sun et al., 2011). It has become a common disease, leading to lipid, protein, and carbohydrate metabolization alterations, favoring pre-disposition to oral complications (Bender & Bender, 2003; Catanzaro et al., 2006; Fouad & Burleson, 2003; Inagaki et al., 2010; Leite et al., 2008, 2010). It has been observed that diabetes mellitus in animal models results in more inflammation of the pulp tissue (Garber et al., 2009), modification in its metabolic response, and alteration in progenitor cell function (Catanzaro et al., 2006; Hata et al., 2015; Inagaki et al., 2010; Kim et al., 2014; Leite et al., 2008, 2010; Nakamura et al., 2011; Tepper et al., 2002). In addition, the hyperglycemic condition has been shown to influence the production of some pro-inflammatory cytokines in oral diseases (Amir et al., 2011; Sun et al., 2011) as well as in pulp tissue (Ferreira et al., 2018).

The relationship between diabetes mellitus and dental diseases have been researched (Bender & Bender, 2003; Catanzaro et al., 2006; Cintra et al., 2014, 2017b; Leite et al., 2008). In a previous study, the pulp tissue of diabetic rats showed higher production of pro-inflammatory cytokines when compared to the tissue of normoglycemic rats after the dental bleaching procedure (Ferreira et al., 2018). IL-6 is considered an anti- and pro-inflammatory cytokine produced during an inflammation process and following the secretion of tumor necrosis factor-alpha (TNF-α) (Schindler et al., 1990), and as TNF-α, it regulates the synthesis of collagenases and prostaglandins (Azuma et al., 2014). Other cytokines act by regulating the expression of pro-inflammatory mediators, such as IL-10 (Li & Flavell, 2008; Moreira et al., 2009). Interleukin IL-10 is an immunosuppressive cytokine capable of acting on the production of pro-inflammatory mediators of the cytokines IL-6 and TNF-α (Li & Flavell, 2008, Moreira et al., 2009), decreasing their expression and moderating damage (Li & Flavell, 2008). Despite some information about the influence of diabetes on the inflammatory process of pulp tissue after dental bleaching, the role of these cytokines over time after bleaching procedure remains unknown.

Differences regarding the reduction of pulp chamber area due to tertiary dentin deposition were also observed in hyperglycemic animals (Cintra et al., 2017a). The hyperglycemic condition caused by diabetes mellitus can influence the defensive and reparative capacity of tissues (Garber et al., 2009; Nagy et al., 2001). It was observed that some growth factors were present considerably higher in the pulp of individuals who had this specific metabolic disorder (Ilić et al., 2012). Transforming growth factors such as TGF-β also play potent regulatory roles, with both TGF-β and IL-10 appearing to regulate pro-inflammatory responses during periapical lesion development in the acute and chronic phases, respectively (Maciel et al., 2012). Together with TGF-β, the fibroblast growth factor FGF-2 seems to be considered a decisive factor in the regenerative and therapeutic process of pulp tissue (Galler et al., 2011; Mathieu et al., 2013; Smith et al., 2016). These findings demonstrate that a better understanding of the effect of diabetes mellitus in pulp tissue is still needed, especially regarding the regenerative process after the bleaching procedure.

Thus, this study aimed to analyze the influence of diabetes mellitus in the inflammatory and regenerative process in the pulp tissue of normoglycemic and diabetic rats. The null hypothesis is that the diabetes does not influence on the inflammatory and regenerative process of the pulp tissue of rats after dental bleaching.

2.3 Materials and methods

Animals

Seventy 2-month-old (200-250 g) male Wistar albino rats were used. Animals were housed in a temperature-controlled environment (22°C ± 1°C, 70% humidity, and a 12 h light-dark cycle) and received water and feed ad libitum. All animal procedures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Bethesda, MD, USA). The experimental protocol was approved by the local Ethics Committee. The anesthetic protocol used before all experimental procedures was by intramuscular injection of ketamine (80 mg kg1, Agener; União Química Farmacêutica Nacional S/A - Embu-Guaçu, SP, Brazil) and xylazine (10 mg kg1, Xilazin, Syntec do Brasil LTDA - Cotia, SP, Brazil).

Induction of Diabetes Mellitus

Thirty-five animals were randomly selected and received a single dose of (35mg kg $^{-1}$) streptozotocin (Sigma-Aldrich Co, Cotia, SP, Brazil), via intravenous injection through the penile vein, diluted in citrate buffer. After 7 days, blood samples were collected (Accu-Chek Performa, Roche Diagnostics Corporation, Indianapolis, IN, USA) from each animal to determine their glycemic levels. The rats with blood glucose levels above 200 mg dl^{-1} were considered hyperglycemic (Cintra et al., 2017a). The other animals received an intravenous injection of citrate buffer to simulate the same procedures.

Dental Bleaching Procedure

After induction of Diabetes Mellitus, the animals were assigned into two main groups (n = 35): normoglycaemic (N) and diabetic (D). The dental bleaching protocol used was based on previous studies (Cintra et al., 2016b, 2017a) and using a concentration of 17.5% hydrogen peroxide (H2O2). To obtain this concentration, a 35% hydrogen peroxide gel (Whiteness HP Maxx, FGM Produtos Odontológicos, Joinville, SC, Brazil) was diluted in distilled water (Soares et al., 2014a, 2014b) in a 3:2:3 proportion, 3 drops of hydrogen peroxide, 2 drops of thickener, and 3 drops of distilled water.

An average of 0.01 mL of 17.5% H2O2 gel was applied with a syringe once to the right upper molars for 30 min and then washed off (Cintra et al., 2016b, 2017a; Soares et al., 2014a, 2014b, 2016). The left molars were used as a control. Thus, a split-mouth design was established after the bleaching procedure, resulting in four experimental hemimaxillae groups: normoglycaemic (N), normoglycaemic-bleached (NBle), diabetic (D) and diabetic-bleached (DBle).

The sample size was established based on previous studies involving pulp tissue analysis in diabetic rats (Ferreira et al., 2018). We considered as 1 the minimum detectable difference in median scores, considering an alpha error of 0.05 and power of 95%, we obtained the number of 7 animals per group.

Laboratory procedures

Immediately (0-hour), 2, 7, 15, and 30 days after bleaching, the animals were euthanized with an overdose of anesthetic solution. The maxillae were separated, dissected, and fixed in 4% buffered formaldehyde for 24 hours and decalcified in 10% EDTA. The tissues were prepared conventionally and embedded in paraffin. Five-micron sections were cut and stained with hematoxylin and eosin (HE) or submitted to immunohistochemistry for IL-6, IL-10, TGF-β, and FGF-2.

For the immunohistochemical reactions, histological sections were deparaffinized in xylene and hydrated in a decreasing series of ethanol. Antigen retrieval was achieved by immersing the histological slides in citrate buffer solution (Antigen Retrieval Buffer, Spring Bioscience, Pleasanton, CA, USA) in a pressurized chamber (Decloaking Chamber; Biocare Medical) at 95 °C for 10 min. The slideswere washed with phosphate-buffered saline (PBS) at the end of each step of the immunohistochemical reaction. Histological sections were immersed in 3% H2O2 solution for 1 h and 20 min and in 1% bovine serum albumin for 12 h to block endogenous peroxidase activity and nonspecific sites, respectively.

Histological slides were split and incubated with one of the following primary antibodies: rabbit anti-IL-6 (1: 100, rabbit anti-IL-6, SC 1265; Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse anti-IL-10 generated rabbit (orb317642, Biorbyt Ltd, Cambridge, UK), mouse anti-TGF-β generated rabbit (orb11468, Biorbyt Ltd) and mouse anti-FGF-2 generated rabbit (LS-C393263, LifeSpan BioSciences, Inc., WA, USA Primary antibodies were diluted in PBS plus 0.1% Triton X-100 (PBS-TX) for 24 hours in a humid chamber. Histological sections were incubated with a biotinylated secondary antibody for 1 h and 30 min and subsequently treated with horseradish streptavidin-peroxidase conjugate for 1 h and 30 min (Dako Labeled Streptavidin-Biotin Universal kit; Dako Laboratories, Carpinteria, CA, USA). The slides were washed with PBS and the reaction was developed with the chromogen 3,30-diaminobenzidine tetrachloride (DAB Chromogen kit; Dako Laboratories, Carpinteria, CA, USA) and was counterstained with Harris hematoxylin. Histological sections were hydrated in decreasing series of ethanol and washed in xylene for subsequent slide mounting.

As a negative control, the specimens were submitted to the procedures described above suppressing the use of primary antibodies. Positive immunolabeling was defined as brownish staining in the cell cytoplasm and extracellular matrix (ECM).

The analysis was performed considering the pulpal horns region at 400x magnification under light microscopy (DM 4000 B, Leica, Wetzlar, Germany). The intensity of inflammation was scored according to the presence of inflammatory infiltrate, being: 1, no or few cells (normal); 2, <25 cells (mild); 3, 25-125 cells (moderate); 4, >125 cells (severe) and 5, (necrosis) (Cintra et al. 2013). The inflammatory reaction was scored according to the average number of inflammatory cells present in the region of the pulp horn of the teeth. Immunohistochemistry was scored using image analysis software (Axiovision 4.8.2® Carl Zeiss, Göttingen, Germany), and the corresponding area was delineated using the color thresholding tool to obtain the optical density of immunolabeling, expressed as percent optical density unit (mean ± standard deviation) (Azuma et al., 2018; Statkievicz et al., 2018).

Statistical analysis

Data were collected and analyzed by a single, calibrated, and blinded operator. Wilcoxon signed-rank test and Mann–Whitney test were used for statistical comparisons of pulp damage, inflammatory response and immunohistochemistry. All statistical analyses were applied at a 5% significance level ($p < 0.05$).

2.4 Results

Inflammatory response

The analysis of the inflammatory process was performed in tissue sections stained in H.E., and immunohistochemistry for IL-6 and IL-10. Representative images of different groups and periods of analysis are shown in Figures 1 to 3. Median scores, interquartile ranges, mean, standard deviation (SD), and p values of Inflammatory infiltrate, and IL-6 and IL-10 percentage of optical density immunostaining (ODI) findings in rats from all groups are shown in Table 1.

In the immediate period (0-hour), it was observed the predominance of necrotic pulp tissue in the bleached groups, regardless of the presence of diabetes. At 2 days, NBle group showed severe inflammation, while the DBle group showed predominance of pulp necrosis ($p < 0.05$) (Fig. 1; Table 1). At 7 days, NBle group showed moderate inflammatory infiltrate, while DBle group showed a severe level of inflammation ($p < 0.05$) (Fig. 1; Table1). At 15 and 30 days, pulp tissue repair was observed, with no areas of pulp necrosis in the NBle and DBle groups ($p > 0.05$) (Fig. 1; Table 1). These groups were associated with the formation of reactionary dentin in the entire pulp chamber as shown in Figure 1. N and D groups showed histological features of the normal pulp tissue, with pre-dentin layer, well-defined and cell-rich acellular layer under intact odontoblastic layer, normal blood vessels, and structured extracellular matrix at all experimental periods as shown in Figure 1.

For IL-6 and IL-10, N and D groups showed similarity in ODI, with no statistically significant difference in all periods ($p > 0.05$). On the other hand, bleached groups showed high ODI for IL-6 and IL-10 in all periods. At 2, 7, and 15 days, the NBle group showed lower ODI for IL-6 when compared to the DBle group ($p < 0.05$) (Fig. 2; Table 1). The higher difference was observed in the 15-day period, where the DBle group showed IL-6 ODI of 68.62% versus 55.07% of the NBle group (Table 1). For IL-10, only at 15 days, the bleaching groups showed differences among themselves, with 27.13% ODI for NBle versus 46.42% ODI of DBle ($p < 0.05$) (Fig. 3; Table 1).

Table 1 The Median Scores, Interquartile Ranges, Mean, Standard Deviation (SD), and p values of Inflammatory infiltrate, and IL-6 and IL-10 percentage of optical density immunostaining (ODI) findings in rats from all groups

Histologic and Immunohistochemical criteria	Periods	Experimental groups				Statistical analysis and p-value
		N	D	NBle	DBle	
Inflammatory infiltrate (median scores and interquartile ranges*)	0-hour	1 (1-1)[a]	1 (1-1)[a]	5 (4-5)[b]	5 (5-5)[b]	NBle x DBle, p = 0.383;
	2 days	1 (1-1)[a]	1 (1-1)[a]	4 (4-5)[b]	5 (4-5)[c]	NBle x DBle, p = 0.031;
	7 days	1 (1-1)[a]	1 (1-1)[a]	3 (2-4)[b]	4 (3-5)[c]	NBle x DBle, p = 0.038;
	15 days	1 (1-1)[a]	1 (1-1)[a]	1 (1-1)[a]	1 (1-1)[a]	NBle x DBle, p = 1;
	30 days	1 (1-1)[a]	1 (1-1)[a]	1 (1-1)[a]	1 (1-1)[a]	NBle x DBle, p = 1;
IL-6 (mean percentage of ODI ± SD*)	0-hour	18.87 ± 3.48[a]	18.43 ± 4.25[a]	50.35 ± 5.09[b]	55.26 ± 7.37[b]	N x D, p = 0.874; NBle x DBle, p = 0.093
	2 days	18.26 ± 3.46[a]	18.09 ± 3.66[a]	58.92 ± 7.26[b]	66.43 ± 3.08[c]	N x D, p = 0.947; NBle x DBle, p = 0.006
	7 days	20.81 ± 3.28[a]	20.75 ± 3.45[a]	56.63 ± 7.72[b]	67.14 ± 3.12[c]	N x D, p = 0.982; NBle x DBle, p < 0.001
	15 days	21.15 ± 4.35[a]	22.38 ± 1.29[a]	55.07 ± 4.68[b]	68.62 ± 5.18[c]	N x D, p = 0.587; NBle x DBle, p < 0.001
	30 days	20.92 ± 3.60[a]	22.27 ± 3.08[a]	51.97 ± 6.15[b]	54.02 ± 5.36[b]	N x D, p = 0.598; NBle x DBle, p = 0.424
IL-10 (mean percentage of	0-hour	21.06 ± 3.64[a]	20.48 ± 2.57[a]	40.24 ± 5.09[b]	40.49 ± 4.92[b]	N x D, p = 0.756; NBle x DBle, p = 0.914

ODI ± SD*)	2 days	23.00 ± 4.33[a]	19.13 ± 3.66[a]	44.89 ± 7.26[b]	44.53 ± 7.12[b]	N x D, p = 0.238; NBle x DBle, p = 0.908
	7 days	20.34 ± 4.28[a]	19.63 ± 3.51[a]	44.18 ± 7.29[b]	43.86 ± 6.71[b]	N x D, p = 0.838; NBle x DBle, p = 0.918
	15 days	20.14 ± 4.47[a]	19.28 ± 8.86[a]	27.13 ± 2.45[b]	46.42 ± 6.44[c]	N x D, p = 0.729; NBle x DBle, p < 0.001
	30 days	18.10 ± 3.68[a]	19.16 ± 3.34[a]	28.42 ± 1.76[b]	28.01 ± 4.09[b]	N x D, p = 0.567; NBle x DBle, p = 0.826

*Different superscript letters represent statistically significant differences in lines ($p < 0.05$)

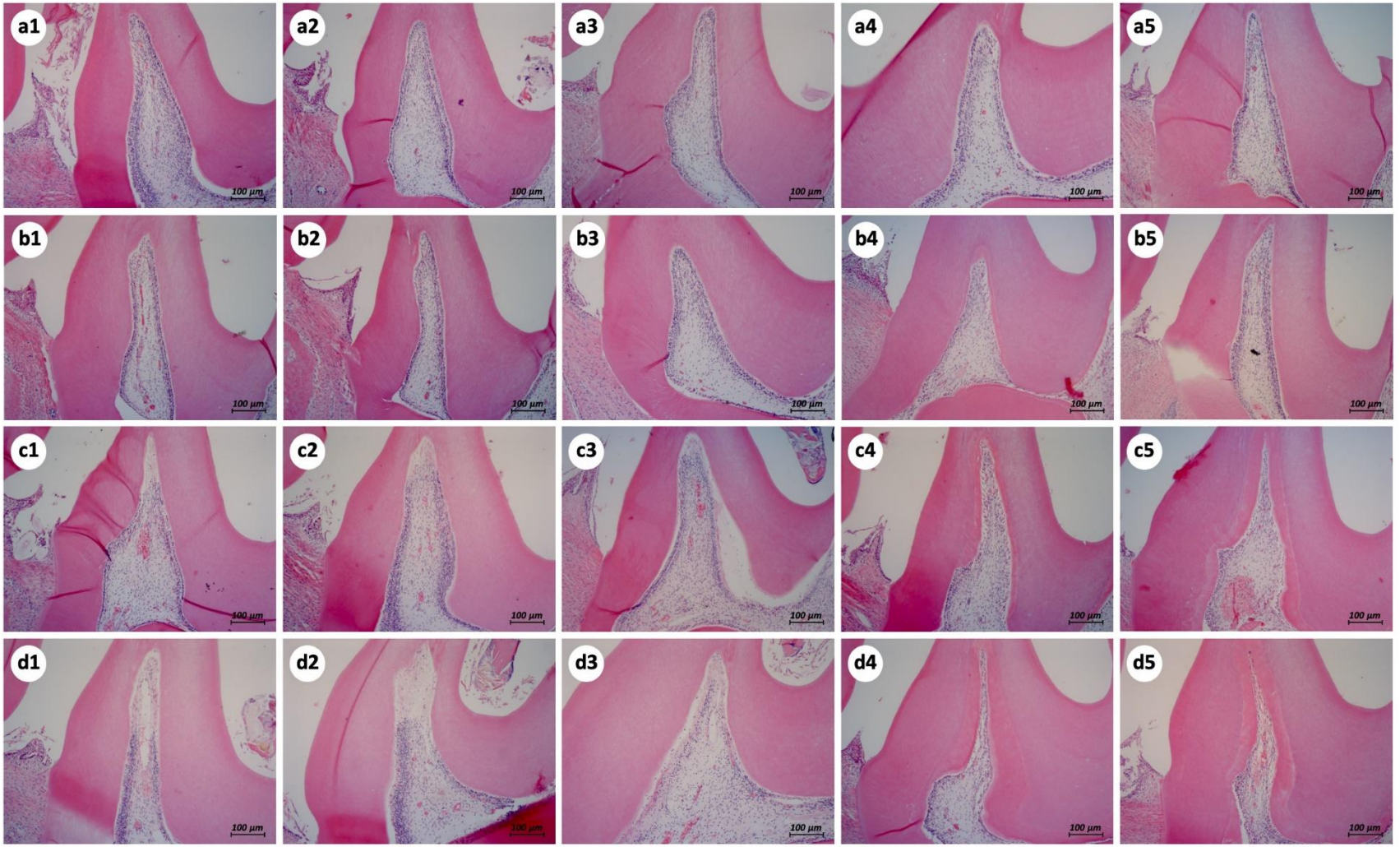

Figure 1 Representative images from HE staining analysis. Groups: N(a), D(b), NBle(c) and DBle(d). Periods: 0-hour (1), 2 days (2), 7 days (3), 15 days (4), and 30 days (5). (HE, 100x).

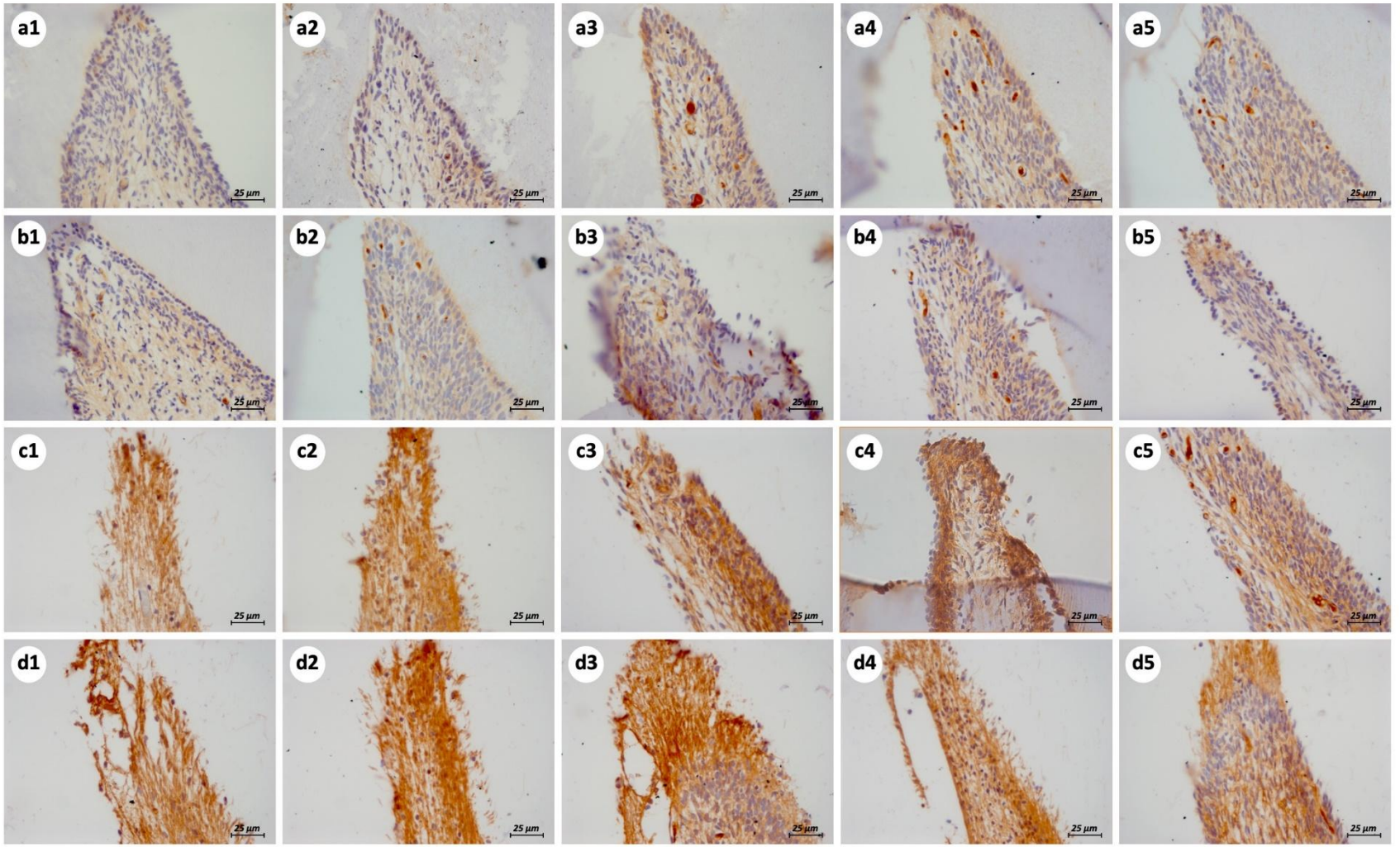

Figure 2 Representative images of IL-6 immunohistochemical analysis. Groups: N(a), D(b), NBle(c) and DBle(d). Periods: 0-hour (1), 2 days (2), 7 days (3), 15 days (4), and 30 days (5). (IL-6, 400x).

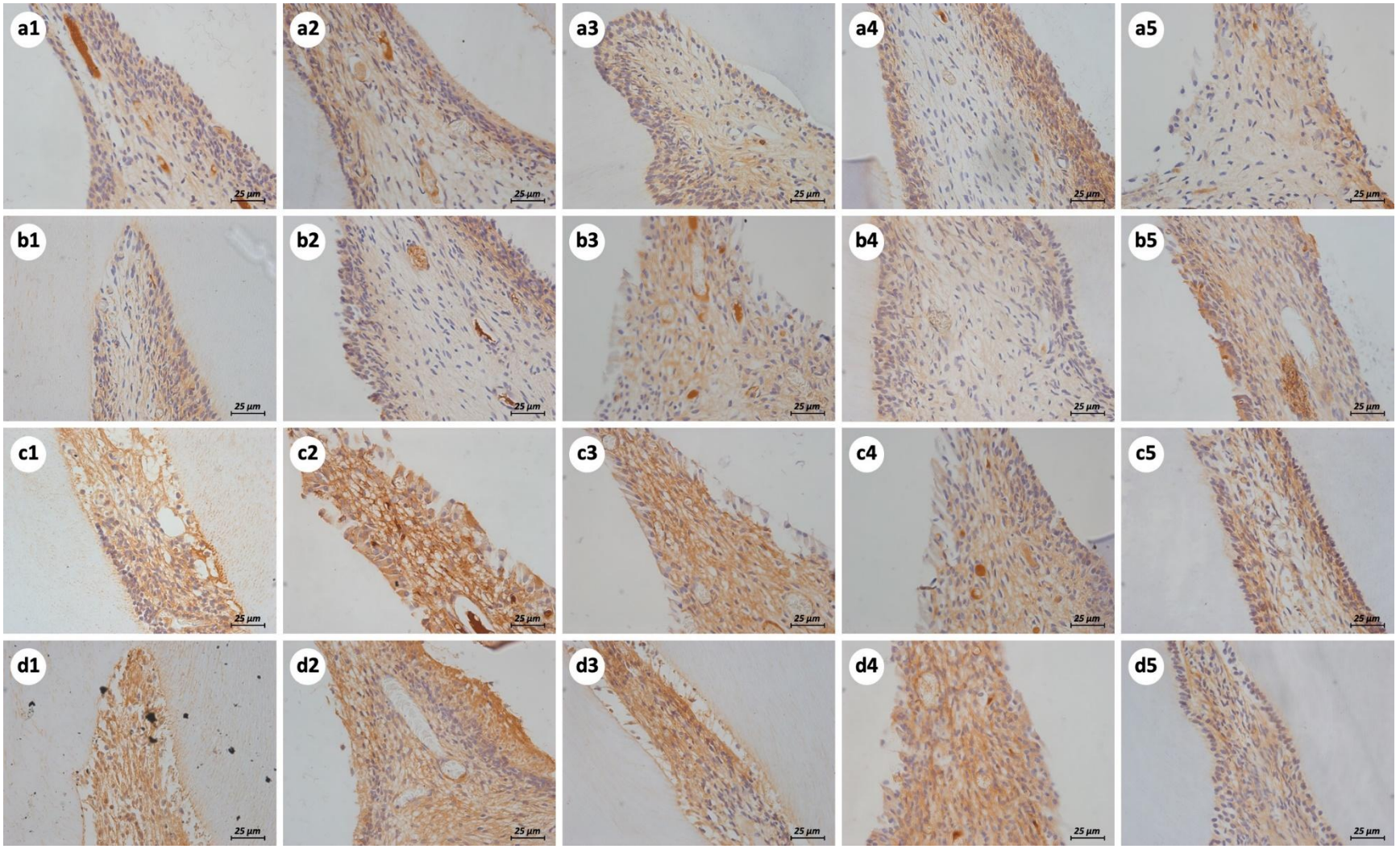

Figure 3 Representative images of IL-10 immunohistochemical analysis. Groups: N(a), D(b), NBle(c) and DBle(d). Periods: 0-hour (1), 2 days (2), 7 days (3), 15 days (4) and 30 days (5). (IL-10, 400x).

Regenerative Process

The regenerative process of the pulp tissue of rats after dental bleaching was observed by immunostaining for transforming growth factors (TGF)-β and fibroblasts (FGF)-2. Representative images of different groups and periods of analysis are shown in Figures 4 and 5. Mean, standard deviation (SD), and p values of percentage of optical density immunostaining (ODI) findings in rats from all groups are shown in Table 2.

N and D groups showed similarity in ODI for TGF-β and FGF-2, with no statistically significant difference in all periods ($p > 0.05$). On the other hand, bleached groups showed high ODI for TGF-β and FGF-2 in all periods (Fig. 4 and 5; Table 2).

At the 0-hour, 7, 15, and 30-day periods, the bleached groups showed similar ODI for TGF-β ($p > 0.05$) (Fig 4). However, at 2 days, the NBle group had a higher ODI with 36.35% versus 10.73% for the DBle group ($p < 0.05$) (Fig. 4; Table 2).

For FGF-2, in 0-hour and 2-days periods, the NBle group showed 34.94% and 35.80% for ODI respectively, while the DBle group 30.76% and 30.93% ODI ($p < 0.05$) (Fig. 5; Table 2). At 7 days the NBle and DBle groups showed similarity of ODI ($p > 0.05$). However, at 15 days, the DBle group outperformed the NBle group showing 36.97% versus 27.52% for ODI ($p < 0.05$). At 30 days the production of FGF-2 was also similar between the groups ($p > 0.05$) (Fig. 5; Table 2).

Table 2 The Mean, Standard Deviation (SD), and p values of TGF-β and FGF-2 percentage of optical density immunostaining (ODI) findings in rats from all groups

Immunohistochemical criteria	Periods	Experimental groups				Statistical analysis and p value
		N	D	NBle	DBle	
TGF-β (mean percentage of ODI ± SD*)	0-hour	6.48 ± 1.21[a]	6.57 ± 2.34[a]	10.50 ± 1.94[b]	10.53 ± 2.18[b]	N x D, p = 0.936; NBle x DBle, p = 0.976
	2 days	6.16 ± 1.19[a]	6.47 ± 2.21[a]	36.35 ± 6.00[b]	10.73 ± 1.90[c]	N x D, p = 0.869; NBle x DBle, p < 0.001
	7 days	7.13 ± 1.70[a]	7.99 ± 1.99[a]	37.06 ± 3.01[b]	36.37 ± 2.71[b]	N x D, p = 0.513; NBle x DBle, p = 0.599
	15 days	5.60 ± 0.99[a]	6.59 ± 1.17[a]	28.48 ± 3.58[b]	30.49 ± 4.56[b]	N x D, p = 0.541; NBle x DBle, p = 0.221
	30 days	7.00 ± 1.64[a]	7.70 ± 1.43[a]	29.01 ± 3.86[b]	28.01 ± 4.20[b]	N x D, p = 0.674; NBle x DBle, p = 0.546
FGF-2 (mean percentage of ODI ± SD*)	0-hour0-hour	10.78 ± 2.41[a]	9.96 ± 1.70[a]	34.94 ± 5.28[b]	30.76 ± 3.96[c]	N x D, p = 0.675; NBle x DBle, p = 0.040
	2 days	11.05 ± 1.78[a]	10.41 ± 1.96[a]	35.80 ± 4.66[b]	30.93 ± 3.65[c]	N x D, p = 0.715; NBle x DBle, p = 0.010
	7 days	10.06 ± 1.33[a]	10.74 ± 1.64[a]	32.94 ± 3.63[b]	35.50 ± 3.18[b]	N x D, p = 0.632; NBle x DBle, p = 0.082
	15 days	10.10 ± 2.42[a]	9.87 ± 2.27[a]	27.52 ± 2.30[b]	36.97 ± 3.89[c]	N x D, p = 0.877; NBle x DBle, p < 0.001
	30 days	11.40 ± 2.35[a]	10.34 ± 1.85[a]	29.08 ± 3.86[a]	28.09 ± 2.85[a]	N x D, p = 0.492; NBle x DBle, p = 0.518

*Different superscript letters represent statistically significant differences in lines ($p < 0.05$)

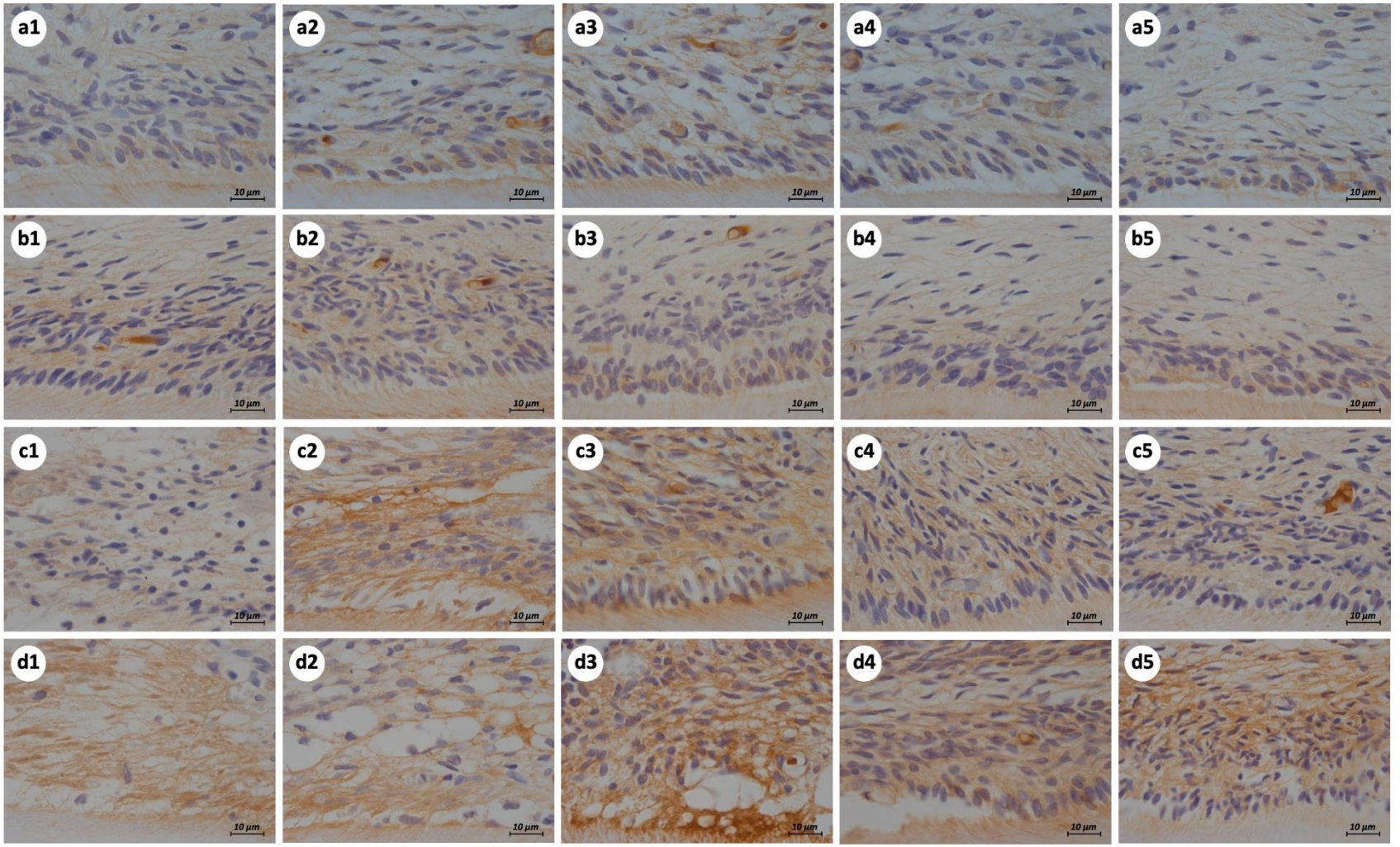

Figure 4 Representative images of immunolabeling for TGF-β. Groups: N(a), D(b), NBle(c) and DBle(d). Periods: 0-hour (1), 2 days (2), 7 days (3), 15 days (4) and 30 days (5). (TGF-β, 1000x).

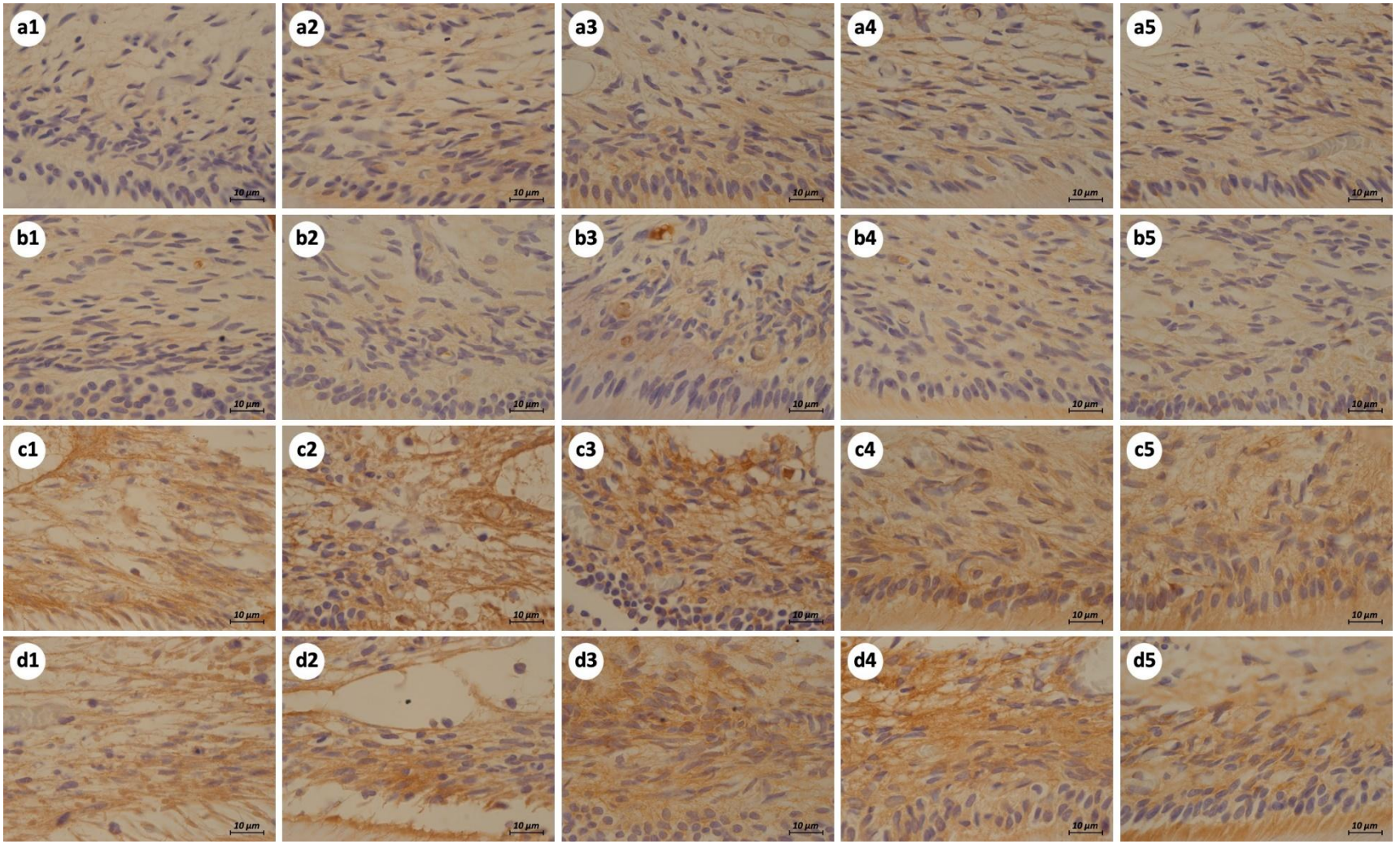

Figure 5 Representative images of immunolabeling for FGF-2. Groups: N(a), D(b), NBle(c) and DBle(d). Periods: 0-hour (1), 2 days (2), 7 days (3), 15 days (4) and 30 days (5). (FGF-2, 1000x).

2.5 Discussion

Although there are physical mechanisms for pulp tissue defense, such as cytoplasmic extensions of odontoblasts and dentinal fluid (Hanks et al., 1993), intensification of the inflammatory process and areas of necrosis were observed after the use of bleaching gel, as bleaching sessions were performed (Cintra et al., 2013). In addition, it has been observed that in the presence of diabetes, the pulp tissue has its responsiveness affected, as well as the performance of progenitor cells (Catanzaro et al., 2006; Hata et al., 2015; Inagaki et al., 2010; Kim et al., 2014; Leite et al., 2008, 2010; Nakamura et al., 2011; Tepper et al., 2002). Thus, the null hypothesis was rejected.

The animal model for dental bleaching has been used in research to evaluate the behavior of the pulp tissue due to its similarity with human teeth regarding anatomical, histological, biological, and physiological characteristics (Cintra et al., 2016b; Dammaschke, 2010; Sasaki & Kawamata-Kido, 1995). The results of the experimental model cannot be extrapolated directly for humans, however, despite limitations such as the lower hardness of the teeth, protocols performed in rats have demonstrated similar results observed in human lower incisors (Barbosa et al., 2020; Costa et al., 2010). Also, the use of animals allows the standardization of the experiment regarding the environment, operative steps and study periods (Cintra et al., 2013, 2016b). Thus, the animal model used is well accepted and can contribute to a better understanding of the changes in the pulp tissue of animals undergoing dental bleaching.

Studies have also shown that bleaching gels with lower concentrations of H2O2 are able to maintain bleaching efficacy and reduce pulpal tissue damage (Benetti et al., 2018b; Cintra et al., 2016a; Soares et al., 2014a, 2014b). In this study, a 17.5% H2O2 concentration was used to bring the animal model used closer to clinical reality and corroborate the already-mentioned protocols. Diabetes mellitus is a disease capable of causing higher predisposition to oral complications that can directly affect the pulp tissue (Bender & Bender, 2003; Catanzaro et al., 2006; Fouad & Burleson, 2003). In this study, histological sections for HE staining were associated with necrotic areas and large inflammatory reactions, with tissue disorganization in

region of the pulp horn. This was especially pronounced in the diabetic bleached group, followed by the normoglycemic bleached group.

The inflammatory process of the pulp tissue is associated with the presence of an intense inflammatory infiltrate and cytokines that may act in the regulation of the immune response during the progression of a disease (Kim & Lim, 2002). The results of the present study revealed that DM associated with dental bleaching induced a significant inflammatory response in the pulp tissue, with necrotic areas in the immediate period and severe inflammatory infiltrate in the initial periods of the NBle and DBle groups, with higher predominance in the diabetic group. Studies have shown that DM can influence the production of pro-inflammatory cytokines in oral diseases (Amir et al., 2011; Sun et al., 2011), with high levels of glucose in the bloodstream being related to increased or decreased pro-inflammatory cytokines, such as interleukin IL-6 (Alexandraki et al., 2006; Wang et al., 2012).

IL-6 is a pleomorphic cytokine found in a network of cytokines that play an important role in specific and nonspecific immune responses (Azuma et al., 2014; Ferreira et al., 2018). Their presence is observed in the progression of inflammation (Nibali et al., 2012), increased vascular permeability of the pulp chamber (Elsalhy et al., 2013; Xiong & Peng, 2015), and contribution to tissue inflammation (Azuma et al., 2014). This was the first study that investigated the behavior of IL-6 in pulpal tissue in normoglycemic and diabetic rats over time. In this study, a higher mean immunostaining optical density was observed for this interleukin in the bleached diabetic groups in all periods when compared to the bleached normoglycemic group. However, this ODI increase was significant at 2, 7 and 15 days for the DBle group when compared to the NBle group. These results may indicate the higher influence of Dm on pulp tissue inflammation in the initial periods of the bleaching treatment, as already observed in a previous study (Ferreira et al., 2018). This cytokine was also observed in the pulp tissue of normoglycemic rats submitted to the bleaching process during the initial periods (Benetti et al., 2018b) and in human cells after the bleaching process (Soares et al., 2015).

Some cytokines are produced by cells that can modulate inflammatory reactions to reduce excessive or unnecessary responses, such as IL-10 (Farges et al., 2015). This cytokine can act on the production of pro-inflammatory mediators of

the cytokines IL-6 and TNF-α (Li & Flavell, 2008; Moreira et al., 2009), decreasing their expression and moderating damage (Li & Flavell, 2008). In this study, in the 15 days, the NBle group showed a lower optical density of immunolabeling for IL-10 when compared to the DBle group. This result may indicate that the DM influenced the maintenance of interleukin production in the diabetic group for a longer period when compared to the normoglycemic group, possibly to reduce the inflammation that was more intense in this group. The expression of IL-10 was also verified in odontoblastic cells in vitro, which may suggest the ability of these cells to regulate the intensity of immune and inflammatory responses in pulp tissue (Farges et al., 2015). At 30 days, the bleached groups showed no further evidence of pulpal inflammation, a result that corroborates with what was observed in previous conducted studies (Benetti et al., 2018a; Cintra et al., 2016b, 2017a; Ferreira et al., 2018).

Dentin is a bioactive tissue with the ability to release growth factors considered fundamental in maintaining pulp tissue therapeutics and regeneration (Galler et al., 2011, Smith et al., 2016). Collagenous and non-collagenous proteins play their roles in the period when dentin tissue formation occurs during tooth development, maintaining calcium ion binding, and controlling cell proliferation and apoptosis (Widbiller et al., 2018). These cellular processes are intensely regulated and reactivated at times of tissue repair (Widbiller et al., 2018), which was already observed in the pulp tissue of teeth undergoing dental bleaching (Benetti et al., 2017). Some of these proteins are transforming growth factor (TGF)-β and fibroblast growth factor (FGF)-2 (Silva et al., 2004; Smith et al., 2016). Thus, the regenerative process of the pulp tissue can be observed, in part, by the production of TGF-β and FGF-2. Changes in these immunomarkers may indicate interference in the reparative process after tissue damage.

TGF-β is an important regulatory cytokine that has effective immunosuppressive effects during inflammatory reactions (Maciel et al., 2012). Studies have observed its action inhibiting the production of pro-inflammatory cytokines, such as TNF-α, as well as antagonizing the biological activities of these cytokines (Colić et al., 2009). In the present study, at 0-hour, 7, 15, and 30 days, the bleached groups, both normoglycemic and diabetic, showed similarity in the optical density of immunolabeling for TGF-β. However, at 2 days, the bleached diabetic group had a lower mean optical density when compared to the bleached

normoglycemic group. This result may indicate that diabetes influenced the production of TGF-β in the initial period, delaying its production, possibly due to the impairment of the pulp tissue caused by the severe inflammation. It was observed that TGF-β can influence the regulation of pulpal inflammatory processes besides acting by stimulating the differentiation of odontoblasts in cases of lesions (Smith 2003), and this process may be compromised in this study by the presence of diabetes mellitus.

Studies have shown that FGF-2 is associated with the in vitro differentiation of pulp cells into odontoblasts (Hao et al., 1999). In this study, it was observed that at 0 hour and 2 days, the diabetic bleached group had lower optical density when compared to the normoglycemic bleached group. This difference disappeared at 7 days and was reversed at 15 days, where the diabetic bleached group started to have a higher immunostaining optical density compared to the normoglycemic bleached group. These results may indicate that diabetes influenced the delayed production of FGF-2 in the pulp tissue of diabetic animals that underwent dental bleaching. The fibroblast growth factor FGF-2 plays a key regenerative role when released after pulp tissue is attacked, acting on the dentin-pulpal complex (Tran-Hung et al., 2008). This could explain the delayed tissue recovery in the diabetic condition that was demonstrated in this study.

This study contributes to the understanding of the inflammatory and regenerative process after bleaching procedures and the influence that diabetes can exert in these cases.

2.6 Conclusion

Diabetes is able to interfere with the severity of pulp tissue inflammation after dental bleaching, increasing IL-6 production and maintaining the production of IL-10 for a longer period, as well as influencing the regenerative process, reducing the production of TGF-β and slowing down the production of FGF-2.

2.7 References

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3 ARTIGO 2 - INFLUENCE OF DIABETES ON THE DEGRADATION OF THE EXTRACELLULAR MATRIX OF THE PULP TISSUE OF RAT TEETH SUBMITTED TO DENTAL BLEACHING‡

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3.1 Abstract

Aim: To evaluate the influence of diabetes mellitus (DM) on the degradation of the extracellular matrix of the pulp tissue of rats after dental bleaching by analyzing the presence of matrix metalloproteinases (MMPs) and metalloproteinase inhibitors (TIMPs).

Methodology: Methodology: Thirty-five Wistar rats received a single dose of (35mg kg $^{-1}$) streptozotocin to induce diabetes (D) and another 35 animals received citrate buffer and were used as controls (C). Dental bleaching with 17.5% H2O2 for 30 min was performed on the upper molars of all animals, forming the groups: C, D, Ble (bleached control) and DBle (bleached diabetic). After 0-hour, 2, 7, 15 and 30 days, the animals were euthanized and the jaws removed and processed for immunohistochemical evaluation for matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9), and for metalloproteinases inhibitors 1 and 2 (TIMP-1 and TIMP-2). Wilcoxon and Mann-Whitney statistical tests were applied ($p<0.05$).

‡ Normalizado de acordo com a Revista “International Endodontic Journal” - https://onlinelibrary.wiley.com/page/journal/13652591/homepage/forauthors.html?1

Results: At 0-hour, 2, 7 and 15 days, Ble and DBle groups showed similarity in immunostaining for MMP-2, MMP-9 and TIMP-1 ($p>0.05$). In the 30-day period, the DBle group presented a higher immunostaining for MMP-2, MMP-9 and TIMP-1 compared to the NBle group ($p<0.05$). Regarding TIMP-2, no differences were found between bleaching groups ($p>0.05$).

Conclusions: It is concluded that diabetes influences the degradation of the extracellular matrix of the pulp tissue after dental bleaching, maintaining for a longer period the production of MMP-2, MMP-9, as well as TIMP-1.

Keywords: dental bleaching; diabetes mellitus; extracellular matrix; matrix metalloproteinases; metalloproteinase inhibitors

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3.2 Introduction

The constituent of bleaching gels responsible for the bleaching capacity is hydrogen peroxide (PH), which once dissociated into reactive oxygen species (ROS), becomes able to diffuse through both enamel and dentin, reacting with components responsible for pigmentation, resulting in dental bleaching (Benetti et al., 2004; Camps et al., 2007, 2010; Carrasco-Guerisoli et al., 2009; Cintra et al., 2016a; Hannig et al., 2011). These ROS are potent oxidizing agents (Camps et al., 2010, Hannig et al., 2011), capable of reaching the dental pulp (Benetti et al., 2017, 2018c; Cintra et al., 2013; Trindade et al., 2009) causing inflammatory infiltrate of different intensities (Benetti et al., 2017, 2018a, 2018b, 2018c; Cintra et al., 2013, 2016b; Ferreira et al., 2018). This pulp tissue reaction occurs due to the cytotoxicity of hydrogen peroxide (Benetti et al., 2018d; Cartagena et al., 2015; Cintra et al., 2016a) and has been shown to be dependent on the concentration of the bleaching agent (Cintra et al., 2016a), the time of application (Cintra et al., 2016b), and the number of the dental bleaching sessions (Cintra et al., 2013).

It is known that some systemic changes can directly affect dental tissues (Jung et al., 2013; Lima et al., 2013). Thus, studies have observed that more intense damage is caused by dental bleaching in specimens with systemic diseases, such as diabetes mellitus (Cintra et al., 2017a; Ferreira et al., 2017). This disease is a metabolic disorder that can cause changes in pulpal tissue structure (Leite et al., 2008), impaired dentinal bridge formation, and increased inflammation of the dental pulp (Garber et al., 2009). This systemic condition when not controlled can generate damage in several organs and tissues, due to oxidative stress and chronic and systemic inflammation resulting from DM (Kajitani et al., 2010; Mirza et al., 2012). Furthermore, it has been observed that diabetes can potentiate the acute phase of inflammation in animal model (Cintra et al., 2017a) and influence the increase of mature collagen fibers after the dental bleaching procedure (Ferreira et al., 2017).

The matrix metalloproteinases (MMPs) have the ability to degrade components found in the extracellular matrix and perform this function in order to allow tissue development, repair, or regeneration (Avădanei et al., 2013; Chang et al., 2017; Cogni et al., 2013; Kim et al., 2012; Roderfeld et al., 2007). The MMP-2 and MMP-9 enzymes are part of the matrix metalloproteinases and act in the degradation of the pulp tissue (Gusman et al., 2002; Shin et al., 2002; Tsai et al., 2005), having part of their activity regulated by inhibitors proteins of metalloproteinases (TIMPs) (Roderfeld et al., 2007). Studies have related TIMP-1 as a specific inhibitor of MMP-9 and TIMP-2 as a modulator of MMP-2 responses (Avădanei et al., 2013; Kim et al., 2012). On the other hand, little is known about the activity of MMPs in the pulp tissue after dental bleaching, as well as the mechanism of regulation of their activity by their respective inhibitors. Particularly, this role in the diabetic condition had not been investigated until the present study.

Thus, the aim of this study was to analyze the influence of diabetes mellitus on the degradation of the extracellular matrix of the pulp tissue of rat teeth submitted to dental bleaching. The null hypothesis is that the diabetes does not influence on the degradation of the extracellular matrix of the pulp tissue of rats after dental bleaching.

3.3 Materials and methods

Animals

Seventy 2-month-old (200-250 g) male Wistar albino rats were used. Animals were housed in a temperature-controlled environment (22°C ± 1°C, 70% humidity, and a 12 h light-dark cycle) and received water and feed ad libitum. All animal procedures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Bethesda, MD, USA). The experimental protocol was approved by the local Ethics Committee. The anesthetic protocol used before all experimental procedures was by intramuscular injection of ketamine (80 mg kg1, Agener; União Química Farmacêutica Nacional S/A - Embu-Guaçu, SP, Brazil) and xylazine (10 mg kg1, Xilazin, Syntec do Brasil LTDA - Cotia, SP, Brazil).

Induction of Diabetes Mellitus

Thirty-five animals were randomly selected and received a single dose of (35mg kg $^{-1}$) streptozotocin (Sigma-Aldrich Co, Cotia, SP, Brazil), via intravenous injection through the penile vein, diluted in citrate buffer. After 7 days, blood samples were collected (Accu-Chek Performa, Roche Diagnostics Corporation, Indianapolis, IN, USA) from each animal to determine their glycemic levels. The rats with blood glucose levels above 200 mg dl^{-1} were considered hyperglycemic (Cintra et al., 2017a). The other thirty-five control animals received an intravenous injection of citrate buffer to simulate the same procedures.

Dental Bleaching Procedure

Dental bleaching with 17.5% H2O2 for 30 min was performed on the upper molars of all animals, forming the groups: Control (C), Bleached (Ble) Diabetic (D) and Bleached Diabetic (DBle).

For dental bleaching procedure, 35% hydrogen peroxide (H2O2) gel (Whiteness HP Maxx, FGM Produtos Odontologicos, Joinville, SC, Brazil) was used, which was diluted in water to obtain a concentration of 17.5% H2O2.

The dilution used a proportion 3:2:3 proportion, 3 drops of hydrogen peroxide, 2 drops of thickener and 3 drops of distilled water.

Then, an average of 0.01 mL of 17.5% H2O2 was applied with a syringe to the right upper molars once for 30 min and then washed off (Cintra et al., 2016b, 2017a; Soares et al., 2014a, 2014b, 2016). The left molars were used as a control.

Laboratory procedures

Immediately (0-hour), 2, 7, 15, and 30 days after bleaching, the animals were euthanized with an overdose of anesthetic solution. The maxillae were separated, dissected, and fixed in 4% buffered formaldehyde for 24 hours and decalcified in 10% EDTA. The tissues were prepared conventionally and embedded in paraffin. Five-micron sections were obtained and submitted to immunohistochemistry for MMP-2, MMP-9, TIMP-1, and TIM-2.

For the immunohistochemical reactions, histological sections were deparaffinized in xylene and hydrated in a decreasing series of ethanol. Antigen retrieval was achieved by immersing the histological slides in citrate buffer solution (Antigen Retrieval Buffer, Spring Bioscience, Pleasanton, CA, USA) in a pressurized chamber (Decloaking Chamber; Biocare Medical) at 95 °C for 10 min. The slides were washed with phosphate-buffered saline (PBS) at the end of each step of the immunohistochemical reaction. Histological sections were immersed in 3% H2O2 solution for 1 h and 20 min and in 1% bovine serum albumin for 12 h to block endogenous peroxidase activity and nonspecific sites, respectively.

Histological slides were split and incubated with one of the following primary antibodies: mouse anti-MMP-2 generated rabbit (LS-C352523, LifeSpan BioSciences); mouse anti-MMP-9 generated rabbit (LS-B10128, LifeSpan BioSciences); mouse TIMP-1 generated rabbit (SC 5538, Santa Cruz Biotechnology, CA, USA) and mouse TIMP-2 generated in rabbit (SC 5539, Santa Cruz Biotechnology).

Primary antibodies were diluted in PBS plus 0.1% Triton X-100 (PBS-TX) for 24 hours in a humid chamber. Histological sections were incubated with a biotinylated secondary antibody for 1 h and 30 min and subsequently treated with horseradish streptavidin-peroxidase conjugate for 1 h and 30 min (Dako Labeled Streptavidin-Biotin Universal kit; Dako Laboratories, Carpinteria, CA, USA). The slides were washed with PBS and the reaction was developed with the chromogen 3,30-diaminobenzidine tetrachloride (DAB Chromogen kit; Dako Laboratories, Carpinteria, CA, USA) and was counterstained with Harris hematoxylin.

Histological sections were hydrated in decreasing series of ethanol and washed in xylene for subsequent slide mounting. As a negative control, the specimens were submitted to the procedures described above suppressing the use of primary antibodies. Positive immunolabeling was defined as brownish staining in the cell cytoplasm and extracellular matrix (ECM).

The analysis was performed considering the pulpal horns region at 400x magnification under light microscopy (DM 4000 B, Leica, Wetzlar, Germany). Immunohistochemistry was scored using image analysis software (Axiovision 4.8.2® Carl Zeiss, Göttingen, Germany), and the corresponding area was delineated using the color thresholding tool to obtain the optical density of immunolabeling, expresse as percent optical density unit (mean ± standard deviation) (Azuma et al., 2018; Statkievicz et al., 2018).

Statistical analysis

The sample size was established based on previous studies involving pulp tissue analysis in diabetic rats (Ferreira et al., 2018). We considered as 1 the minimum detectable difference in median scores, considering an alpha error of 0.05 and power of 95%, we obtained the number of 7 animals per group per time period.

Data were collected and analyzed by a single, calibrated, and blinded operator. Wilcoxon signed-rank test and Mann–Whitney test were used for statistical comparisons of pulp damage, inflammatory response and immunohistochemistry. All statistical analyses were applied at a 5% significance level ($p < 0.05$).

3.4 Results

The process of extracellular matrix degradation of rat pulp tissue after dental bleaching was observed by immunolabeling for metalloproteinases markers MMP-2 and MMP-9 and metalloproteinases inhibitors TIMP-1 and TIMP-2. Representative images of different groups and analysis periods are shown in Figures 1, 2, 3 and 4 respectively. Mean, standard deviation (SD), and p values of percentage of optical density immunostaining (ODI) findings in rats from all groups are shown in Table 1.

At 0-hour, 2, 7, and 15 days, the Ble and DBle groups showed similarity in the optical density of immunolabeling for MMP-2 ($p>0.05$) (Figure 1; Table 1). However, at 30 days, the DBle group had a higher optical density of immunostaining with 16.96% against 13.41% of the Ble group ($p<0.001$).

Regarding the immunostaining of MMP-9, the Ble and DBle groups showed similarity of optical density in the periods of 0-hour, 2, 7, and 15 days ($p>0.05$). In the 30-day period, the immunolabeling of the diabetic bleached group showed superiority compared to the control bleached group, with percentages of 35.19% and 28.48%, respectively ($p<0.001$) (Figure 2; Table 1). The results for TIMP-1 were also concentrated in the 30 days, with 25.24% of optical density immunolabeling in the DBle group, versus 20.72% in the Ble group ($p = 0.011$) (Figure 3; Table 1). In the periods 0-hour, 2, 7, and 15 days the groups C, D, Ble, and DBle, showed similarity in optical density ($p>0.05$).

In all periods analyzed for TIMP-2 immunostaining, the results showed similarity in optical density for the Ble and DBle groups, without statistically significant differences ($p>0.05$) (Figure 4; Table 1).

Table 1 The Mean, Standard Deviation (SD), and p values of MMP-2, MMP-9, TIMP-1 and TIMP-2 percentage of optical density immunostaining (ODI) findings in rats from all groups

Immunohistochemical criteria	Periods	Experimental groups				Statistical analysis and p value
		C	D	BIe	DBIe	
MMP-2 (mean percentage of ODI ± SD*)	0-hour	12.48 ± 2.04a	12.79 ± 2.25a	16.12 ± 2.19b	17.08 ± 2.14b	C x D, p = 0.791; NBIe x DBIe, p = 0.414
	2 days	12.51 ± 2.01a	12.50 ± 1.61a	16.41 ± 2.10b	17.06 ± 0.94b	C x D, p = 0.999; NBIe x DBIe, p = 0.489
	7 days	12.83 ± 1.31a	13.78 ± 1.78a	17.81 ± 1.80b	17.52 ± 1.45b	C x D, p = 0.276; NBIe x DBIe, p = 0.736
	15 days	11.79 ± 1.76a	12.04 ± 1.89a	17.70 ± 2.04b	17.29 ± 1.74b	C x D, p = 0.800; NBIe x DBIe, p = 0.683
	30 days	13.17 ± 1.76a	12.88 ± 1.98a	13.41 ± 1.62a	16.96 ± 1.89b	C x D, p = 0.766; NBIe x DBIe, p = 0.001
MMP-9 (mean percentage of ODI ± SD*)	0-hour	2.58 ± 1.23a	2.55 ± 1.16a	27.50 ± 5.76b	25.56 ± 6.85b	C x D, p = 0.993; NBIe x DBIe, p = 0.434
	2 days	2.73 ± 0.74a	3.10 ± 0.65a	29.82 ± 4.59b	28.63 ± 3.28b	C x D, p = 0.813; NBIe x DBIe, p = 0.445
	7 days	2.27 ± 0.83a	2.10 ± 1.04a	39.03 ± 4.96b	37.57 ± 4.89b	C x D, p = 0.932; NBIe x DBIe, p = 0.450
	15 days	2.49 ± 1.42a	3.04 ± 1.00a	36.25 ± 4.53b	34.23 ± 4.89b	C x D, p = 0.768; NBIe x DBIe, p = 0.284
	30 days	2.49 ± 1.03a	2.47 ± 0.56a	28.48 ± 4.53b	35.19 ± 5.18c	C x D, p = 0.990; NBIe x DBIe, p = 0.001
TIMP-1 (mean percentage of ODI ± SD*)	0-hour	7.03 ± 1.53a	6.67 ± 1.85a	19.10 ± 4.53b	20.93 ± 3.56b	C x D, p = 0.831; NBIe x DBIe, p = 0.284
	2 days	6.97 ± 1.47a	7.04 ± 0.68a	19.73 ± 3.60b	20.87 ± 4.11b	C x D, p = 0.962; NBIe x DBIe, p = 0.459
	7 days	5.93 ± 1.21a	6.38 ± 1.48a	17.00 ± 4.46b	18.82 ± 2.23b	C x D, p = 0.757; NBIe x DBIe, p = 0.214
	15 days	6.87 ± 1.57a	6.08 ± 1.42a	17.75 ± 3.45b	18.57 ± 5.51b	C x D, p = 0.672; NBIe x DBIe, p = 0.656
	30 days	6.82 ± 1.33a	6.17 ± 1.22a	20.72 ± 4.71b	25.24 ± 3.51c	C x D, p = 0.696; NBIe x DBIe, p = 0.011
TIMP-2 (mean percentage	0-hour	6.08 ± 1.18a	6.39 ± 0.89a	24.04 ± 4.12b	21.46 ± 3.60b	C x D, p = 0.837; NBIe x DBIe, p = 0.101

of ODI ± SD*)	2 days	5.68 ± 0.60a	6.18 ± 1.49a	22.24 ± 2.36b	24.81 ± 3.88b	C x D, p = 0.701; NBle x DBle, p = 0.057
	7 days	5.77 ± 1.22a	6.53 ± 1.69a	41.14 ± 3.94b	39.86 ± 4.15b	C x D, p = 0.643; NBle x DBle, p = 0.437
	15 days	6.03 ± 1.25a	6.23 ± 1.73a	31.26 ± 3.72b	31.79 ± 5.46b	C x D, p = 0.916; NBle x DBle, p = 0.776
	30 days	6.51 ± 1.17a	7.14 ± 1.79a	29.49 ± 7.50b	28.15 ± 5.70b	C x D, p = 0.809; NBle x DBle, p = 0.609

*Different superscript letters represent statistically significant differences in lines ($p < 0.05$)

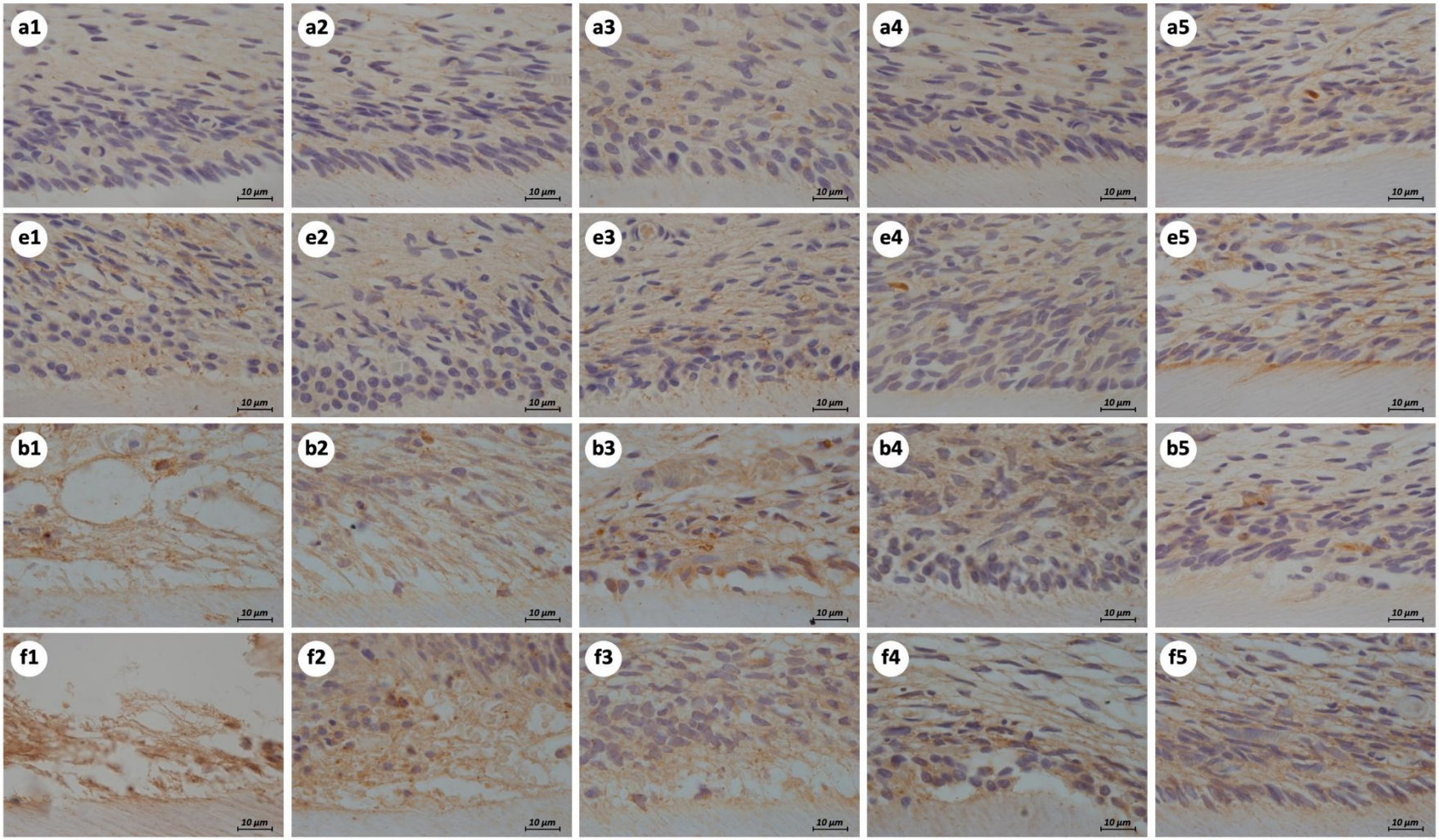

Figure 1 Representative images of MMP-2 immunohistochemical analysis. Groups: C(a), D(b), Ble(c) and DBle(d). Periods: 0-hour (1), 2 days (2), 7 days (3), 15 days (4) and 30 days (5). (MMP-2, 400x).

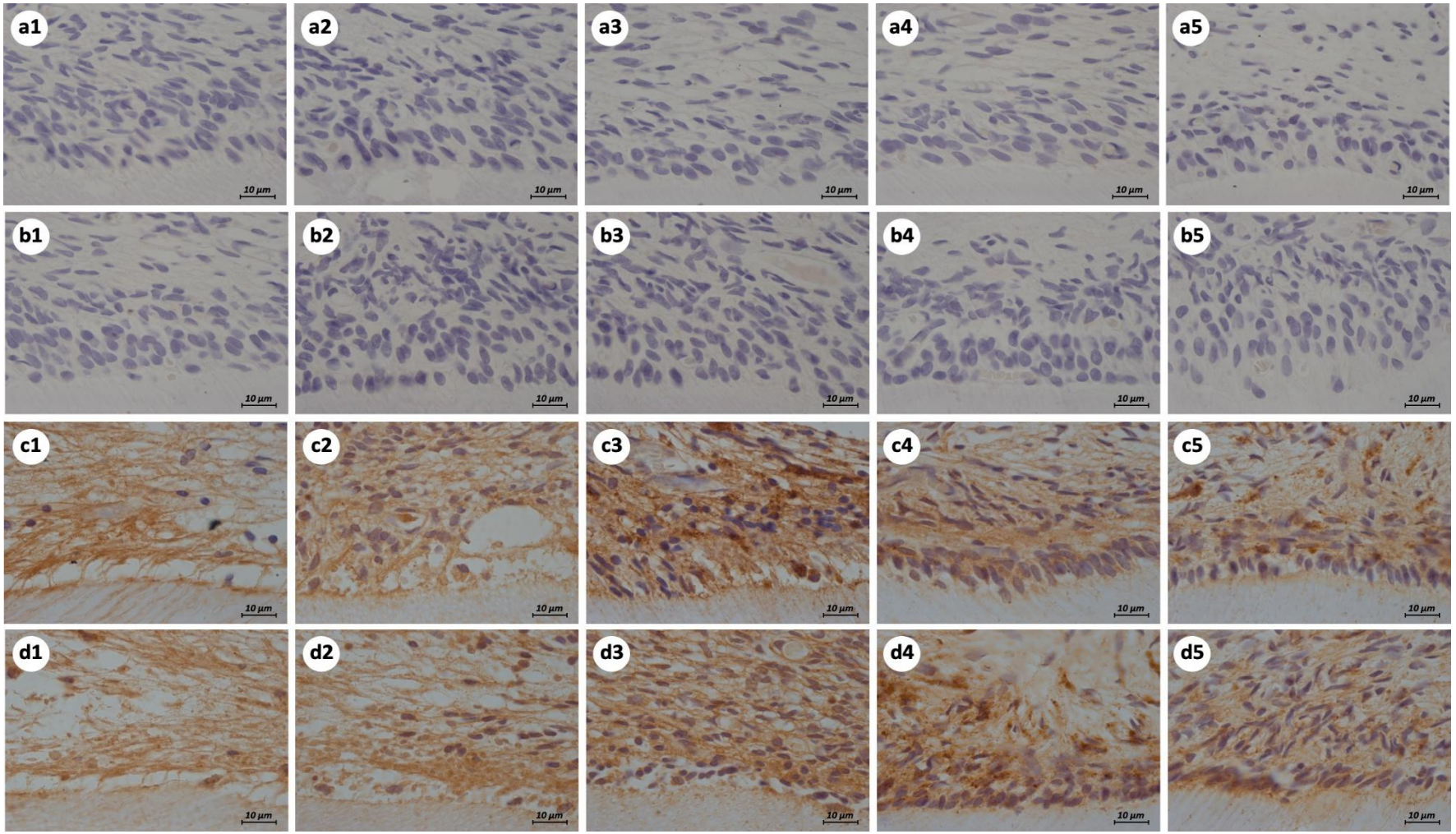

Figure 2 Representative images of MMP-9 immunohistochemical analysis. Groups: C(a), D(b), Ble(c) and DBle(d). Periods: 0-hour (1), 2 days (2), 7 days (3), 15 days (4) and 30 days (5). (MMP-9, 400x).

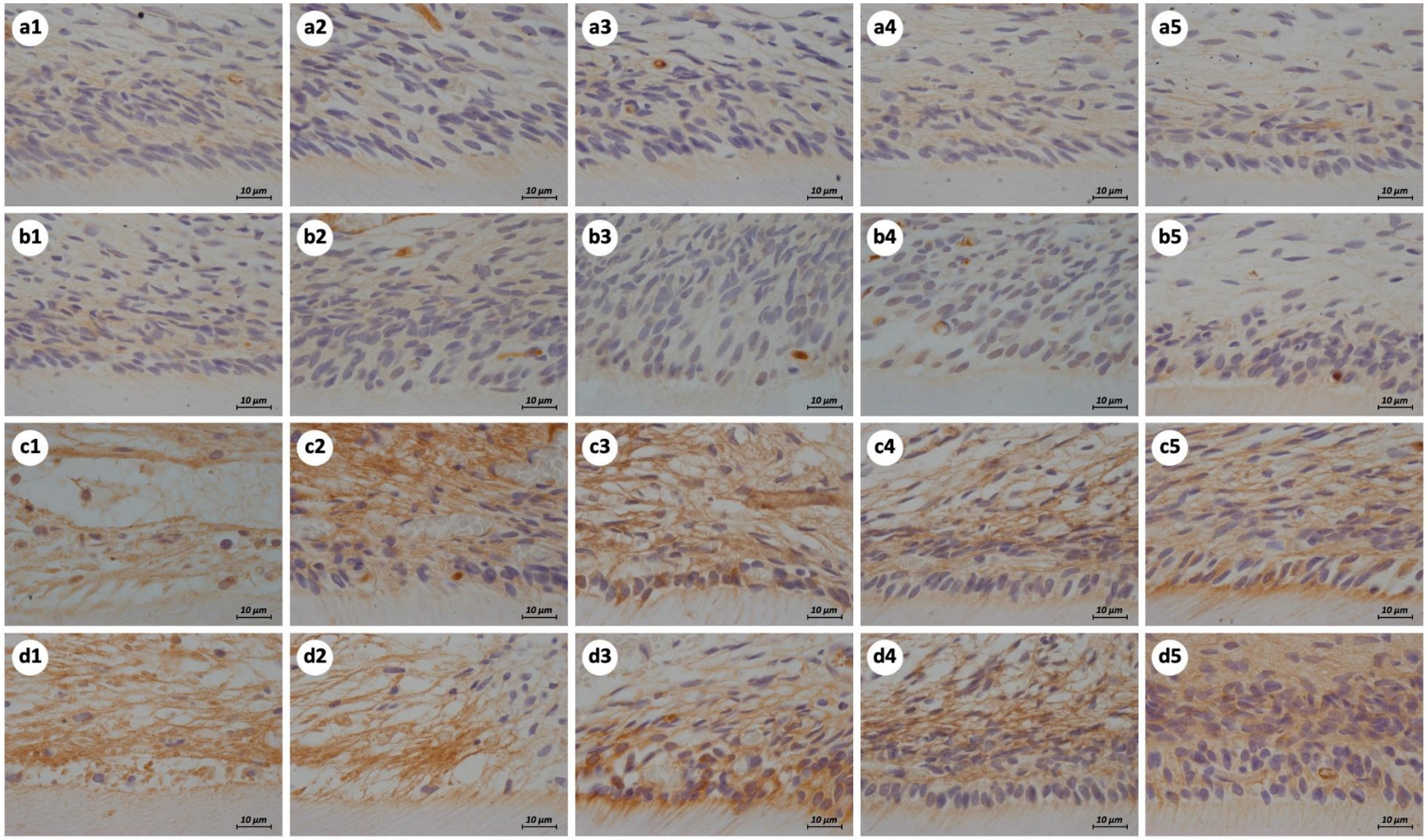

Figure 3 Representative images of TIMP-1 immunohistochemical analysis. Groups: C(a), D(b), Ble(c) and DBle(d). Periods: 0-hour (1), 2 days (2), 7 days (3), 15 days (4) and 30 days (5). (TIMP-1, 400x).

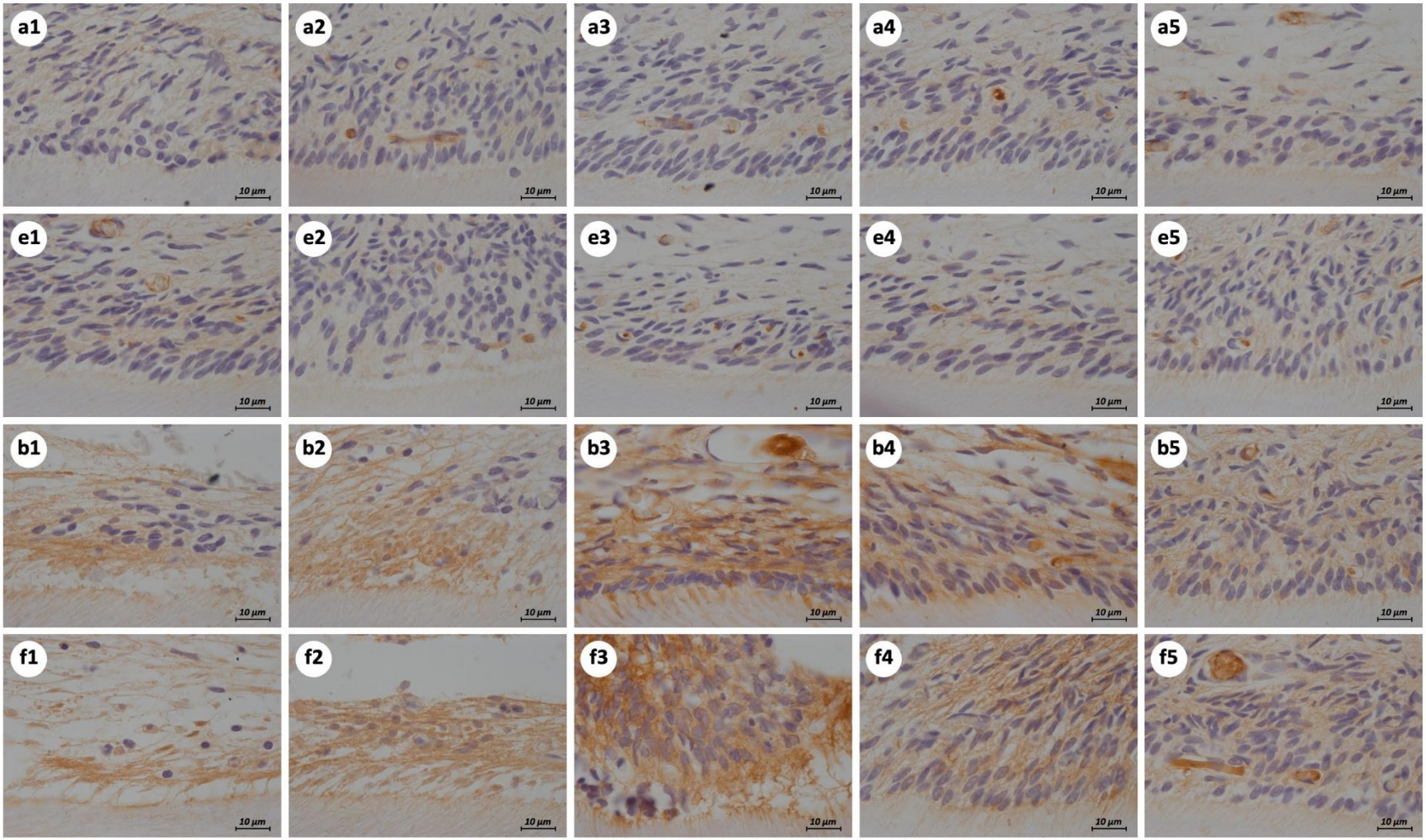

Figure 4 Representative images of TIMP-2 immunohistochemical analysis. Groups: C(a), D(b), Ble(c) and DBle(d). Periods: 0-hour (1), 2 days (2), 7 days (3), 15 days (4) and 30 days (5). (TIMP-2, 400x)

3.5 Discussion

In this study, diabetes influenced the degradation of the extracellular matrix of the pulp tissue after dental bleaching, rejecting the null hypothesis. Due to the ability to induce changes to the dentin-pulp complex, the effects of bleaching gel on the dental pulp have been studied (Cintra et al., 2013, 2016a; Costa et al., 2010; Ferreira et al., 2013; Seale et al., 1981). When in contact with pulp tissue, cells morphological alterations (Coldebella et al., 2009; Trindade et al., 2009), inflammation and decreased in cellularity and cellular metabolism (Cintra et al., 2013; Min et al., 2008; Seale et al., 1981; Soares et al., 2014a) are some of the damages that have already been observed. Besides being associated with morphological changes in mineralized dental tissues (Bistey et al., 2007; Fattibene et al., 2005), the effect of dental bleaching has been analyzed in previous studies of the group, especially in diabetic specimens, with significant results (Cintra et al., 2017a; Ferreira et al., 2018).

Diabetes mellitus (DM) is characterized by a hyperglycemic condition that causes changes in the metabolization of lipids, proteins, and carbohydrates (Sun et al., 2011). The relationship established between this disease and endodontic changes has been observed in several works (Cintra et al., 2014a, 2014b, 2017b; Ferreira et al., 2017; Prieto et al., 2017). Studies have shown that in the presence of diabetes, the pulp tissue becomes more predisposed to pathological calcification (Inagaki et al., 2010), exacerbated inflammation (Ferreira et al., 2018), and inhibited cell differentiation capacity (de Azevedo-Queiroz et al., 2018). Also, it has been observed that the hyperglycemic condition causes a reduction in collagen concentration and increased alkaline phosphatase activity (Catanzaro et al., 2006). However, even after all these findings, the effect of diabetes mellitus on the extracellular matrix in specimens submitted to dental bleaching had not been investigated until the present study.

The stages of tissue development, repair, and regeneration are directly related to the renewal of extracellular matrix collagen, a process that is regulated by proteolytic enzymes (Avădanei et al., 2013; Chang et al., 2017; Cogni et al., 2013; Kim et al., 2012; Roderfeld et al., 2007), such as the metalloproteinases MMP-2 and MMP-9. MMPs are known to degrade matrix macromolecules, and together they are able to degrade all the component proteins of the ECM and basement membranes

(Birkedal-Hansen, 1993). In the present study, in the period of 30 days, it was possible to observe that the optical density of immunolabeling for MMP-2 was elevated in the group of diabetic rats compared to the normoglycemic group. This result may indicate that diabetes influenced the maintenance of MMP-2 production for a longer experimental time. Studies have shown that MMPs are involved in pulp tissue destruction (Gusman et al., 2002; Shin et al., 2002; Tsai et al., 2005), in this finding, diabetes may have accentuated this response.

MMP-9 or gelatinase B, like MMP-2, is an important metalloproteinase that has been observed in the formation and mineralization of rat dental tissues in previous studies (Fanchon et al., 2004). Furthermore, its presence was found to be more significant in inflamed pulps as a result of pulpitis, which may suggest the action of this metalloproteinase in tissue breakdown in situations of pulp inflammation (Gusman et al., 2002). In this study, the greatest difference between the groups occurred during the 30-day period, in which the optical density of immunolabeling in the diabetic group was higher when compared to the normoglycemic group. This finding may indicate that diabetes mellitus influences the maintenance of MMP-9 production in the pulp tissue for a longer period of time.

The performance of MMP-9 is regulated by the specific inhibitor TIMP-1, and the result of the interaction between both, is the balance in the regulation of activation and functionality of the metalloproteinase when it acts in the tissue (Kim et al., 2012). In the present study, TIMP-1 expression was shown to be higher in the 30 days, demonstrated by the mean optical immunolabeling density of the DBle group which was higher than the Ble group. This result may indicate the influence of diabetes in maintaining the inhibitor for a longer time, possibly modulating the expression of MMP-9 in this period, since the findings also showed higher expression of this metalloproteinase. The harmonious relationship between MMPs and TIMPs is considered to be responsible for extracellular matrix turnover in damaged pulp tissue (Wisithphrom et al., 2006).

Likewise TIMP-1 which plays a regulatory role on MMP-9, TIMP-2 has been shown to be a modulator for MMP-2 activity (Kim et al., 2012, Avădanei et al., 2013). In this study, the behavior of TIMP-2 according to the immunostaining optical density was similar in all experimental periods, regardless of the presence of diabetes. This

finding may indicate that diabetes exerts higher influence on the MMP-9 and TIMP-1 pathway in relation to the MMP-2- and TIMP-2 pathway.

Studies with direct evidence of the relationship between MMPs and pulp tissue are still scarce, as well as their modulators are known as TIMPs. However, the exposed findings are partly supported by studies already carried out and allow significant findings on the interrelation between diabetes, dental bleaching, MMPs, and TIMPs.

3.6 Conclusion

Diabetes is able to influence the degradation of the extracellular matrix of the pulp tissue after dental bleaching, maintaining for a longer period the production of MMP-2, MMP-9, as well as TIMP-1.

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4 CONCLUSÃO GERAL

Em conclusão, a diabetes influencia na severidade da inflamação do tecido pulpar após clareação dentária, elevando a produção de IL-6 e mantendo por maior período a produção de IL-10, assim como influencia no processo regenerativo, reduzindo a produção de TGF-β e retardando a produção de FGF-2. Ainda, influencia na degradação da matriz extracelular, mantendo elevada por maior período a produção de MMP-2 e MMP-9, assim como de TIMP-1.

ANEXOS

ANEXO A - Referências da Introdução Geral

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ANEXO B – Comissão 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 **"Influência da diabetes no tecido pulpar de dentes de ratos submetidos à clareação dentária"**, Processo FOA nº 0508-2022, 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 30 de Novembro de 2022.

VALIDADE DESTE CERTIFICADO: 09 de Dezembro de 2023.
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 09 de Janeiro de 2024.

CERTIFICATE

We certify that the study entitled **"Influence of diabetes on pulp tissue of rat teeth submitted to tooth bleaching"**, Protocol FOA nº 0508-2022, 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 30, 2022.

VALIDITY OF THIS CERTIFICATE: December 09, 2023.
DATE OF SUBMISSION OF THE FINAL REPORT: January 09, 2024.

Prof. Dr. Fellippo Ramos Verri
Coordenador da CEUA
CEUA Coordinator

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