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Increased Pulp Degeneration and HIF-1α Expression In Teeth With Severe Periodontitis

Aumento de la Degeneración Pulpar y Expresión de HIF-1α en Dientes con Periodontitis Grave

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SUMMARY: Studies on the long-term effects of chronic periodontitis on pulp degeneration are still incomplete. Seventy-four extracted teeth suffered from severe periodontitis were selected to provide insights for the assessment of dental pulp status. Tissue slices were prepared using HE staining to observe pathological changes in the pulps. Typical pulp degeneration lesions were selected to observe the expression of HIF-1α by immunohistochemistry, immunofluorescence and western blotting. Pulp degeneration was not observed in the normal control teeth. Twenty-two pulp degeneration was identified among the 74 teeth with severe periodontitis(30 %). Positive expression of HIF-1α was observed in odontoblasts, degenerated pulp cells, and proliferative fibroblasts. The normal dental pulp showed minimal positive expression. Western blotting analysis revealed a significant elevation in HIF-1α expression in teeth affected by severe periodontitis compared to the normal group. The dental pulp of teeth affected by periodontitis is more prone to pulp degeneration, with HIF-1α involved in this process.

KEY WORDS: Periodontitis; Pulp degeneration; HIF-1α; Periodontal-endodontic combined disease; Calcification.

INTRODUCTION

The dental pulp is the soft tissue located at the center of the tooth, containing blood vessels, nerves, and other cells. Pulp degeneration refers to the regressive changes or degeneration of dental pulp, such as fibrosis, calcifications, vacuolar degeneration and reticular atrophy. When the tooth is subjected to damage, infection, or other adverse factors, the dental pulp tissues may undergo regressive changes, leading to the loss of its structure and function. Pulp degeneration can result in tooth sensitivity, pain, infection, and other clinical issues(Tan *et al*., 2020).

Periodontitis ranks among the most common oral diseases in humans. In recent years, the impact of periodontal diseases on dental pulp tissues has garnered recognition among the majority of scholars (Siqueira & Rôças, 2022). Several studies have suggested a potential link between untreated periodontal diseases and alterations in dental pulp tissues. Scholars (Guo *et al*., 2022) have conducted studies on periodontal pockets and root canals and found a high similarity in bacterial flora between the two, confirming the relationship between periodontal tissues and dental pulp bacterial infections. Toxic products produced by microorganisms and periodontal inflammatory environment may infiltrate dental pulp, which may lead to disruption of homeostatic maintenance (Koca-Ünsal *et al*., 2022; Arias *et al*., 2023). However, research on the correlation between periodontitis and pulp degeneration remains relatively scarce, especially its related mechanisms.

Extracted teeth suffered from severe periodontitis were useful to assess dental pulp status. Early histological investigations have indicated that minimal changes are observed in pulp tissues in the majority of cases associated with moderate and advanced periodontitis (Ricucci *et al*.,

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2021). Sabeti *et al*. (2021) conducted histopathological evaluations of teeth affected by severe periodontitis and concluded that periodontal diseases do not significantly impact pulp vitality. However, Wan *et al*. (2015) examined histological sections to assess the relationship between periodontitis and pulpal lesions, revealing a positive correlation between the severity of periodontal diseases and histological alterations within the pulp. Studies from Xiao *et al*. (2020) have reported changes in the expression and distribution of HIF-1α during early pulp inflammation, providing evidence of HIF-1α's regulatory role in dental pulp inflammation. It is crucial to verify whether the hypoxic environment present in periodontitis teeth further exacerbates hypoxia in the pulp, ultimately leading to dental pulp lesions.

This study aimed to investigate pathological alterations in the dental pulp and the expression of HIF-1α in 74 teeth affected by severe periodontitis. The goal was to elucidate the impact of periodontitis on pulp degeneration, offering novel insights for the management of severe periodontitis, as well as the diagnosis and treatment of periodontal-endodontic combined diseases.

MATERIAL AND METHOD

Cases Selection and Experimental Grouping. Seventy-four teeth (43 anterior teeth, 18 premolars, and 13 molars) were extracted due to severe periodontitis at Jinan Stomatological Hospital from February 2021 to February 2022. Severe periodontitis was diagnosed based on the guidelines provided by the American Academy of Periodontology (1999 International International Workshop for a Classification of Periodontal Diseases and Conditions, 1999). All specimens exhibited complete root development without any filling materials, restorations, caries, or cracks. Additionally, premolars with complete root formation and no evident caries or defects were extracted as a control group for orthodontic treatment (1999 International International Workshop for a Classification of Periodontal Diseases and Conditions, 1999). All teeth used in this study were obtained post-extraction with informed consent from the patients.

Patient selection criteria. Patients aged 22 to 80 years, with an average age of 59 years, were included if they had no systemic medical history such as hypertension, heart disease, or diabetes and exhibited no malocclusion (including crowding, deep overjet, or deep overbite), bruxism, clenching habits, or habits of biting hard objects. Additionally, patients should not have received periodontal treatment for at least one year prior to the study.

Tooth selection criteria. Teeth were selected based on having complete crowns and roots, complete dental development, no history of dental pulp treatment, no significant decay or micro-cracks, no apparent developmental abnormalities, and no previous occurrence of apical periodontitis. For the experimental group (Group I), teeth were chosen based on X-rays indicating apical bone resorption reaching the apical third or more of the root length, absence of root apical shadow, and teeth with mobility degrees II-III. The control group (Group II) consisted of healthy teeth extracted for orthodontic reasons without bone resorption.

Specimen Handling. Freshly extracted teeth were fixed in 4 % formaldehyde solution for 72 hours, numbered, rinsed, and soaked in 15 % EDTA decalcification solution with daily solution changes at room temperature until teeth softened to allow the passage of an expanding needle without resistance (approximately 4N). Subsequently, teeth were rinsed for about 12 hours to remove residual decalcifying solution, followed by embedding in paraffin. Sections were stained with hematoxylin, eosin and immunohistochemical staining. Two independent pathologists who were unaware of the clinical information assessed the sections. In cases of disagreement, a third examiner was consulted.

Histopathological Observation. Three slices of each root containing complete apical root pulp were obtained for HE staining to document pathological changes in the pulp tissue. Sections were first deparaffinized using xylene, hydrated in gradient ethanol and subjected to hematoxylin for 3 minutes to stain the cell nucleus. Following a five-minute wash with running water, the sections were reacted with eosin for 30 seconds.

Immunohistochemical staining and immunofluorescence. A Streptavidin-Peroxidase kit (Zhongshan, Beijing, China) was used strictly following the manufacturer's instructions. The polyclonal antibody HIF-1α (1:100, Proteintech, USA) was used for immunohistochemical staining and immunolfuorescence. Nuclei were stained with a DAPI solution.

Western blotting analysis. The MinuteTM Total Protein Extraction Kit (Inventbiotech, Plymouth, MN, USA) was used to extract total proteins. Briefly, 10 % SDS-PAGE was used to separate equal proteins, which were subsequently electroblotted to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5 % nonfat milk, the primary antibodies HIF-1α (1:500, Abcam, UK) and β-actin (1:500, Servicebio) were added and incubated overnight at 4 °C. Horseradish peroxidase-goat anti-rabbit IgG (1:10000; CWBiotech, Beijing, China) was incubated at room temperature for 1 h. Finally, the blots were visualized with an Amersham Imager 800 instrument.

Statistical Analysis. All data were statistically executed using SPSS 23.0 software. Chi-square t-tests were used to compare the two different groups. Statistical significance was considered as a p-value of less than 0.05.

RESULTS

Observation of dental pulp pathological changes. A series of pathological changes such as fibrosis, hyperemia, inflammation, calcification, reticular atrophy, and vacuolar degeneration of the odontoblasts layer can be observed in the teeth affected by severe periodontitis, with some pulp even showing signs of necrosis In the experimental group, necrotic pulps accounted for 40.54 %, followed by degenerative pulps at 29.73 %, inflammatory pulps at 17.57 %, normal pulp at 10.81 %, and hyperemia at 1.35 %. In contrast, the control group predominantly exhibited normal pulp (90.00 %), with hyperemia accounting for 10.00 % (Table I).

Observation of dental pulp degeneration. In this study, 22 teeth were classified as pulp degeneration. Four different pathological changes of the pulp degeneration were observed.

Increased fibrous components are increased in the pulp fibrosis (Fig. 1 A1). Large collagen fibers parallel to the dental pulp's longitudinal axis were observed (black arrows) (Fig. 1 A2). HE staining showed blue-stained granular masses (black arrows) in pulp calcification (Figs. 1 B1 and B2). Vacuolar degeneration of the odontoblastic layer: A decrease in the volume of odontoblasts is apparent, with intercellular vesicles causing them to be compacted into clusters resembling "straw bundles" (black arrows) (Figs. 1C1 and C2). Reticular atrophy of the pulp: Fluid accumulation within and between pulp cells results in the presentation of pulp tissue with vacuolated spaces of various sizes filled with fluid. Odontoblasts decrease in number, and the pulp tissue exhibits a "reticular" appearance. Spaces of varying sizes resembling vesicles are observed within the dental pulp tissue (black arrows) (Figs. 1D1 and D2).

E**xpression of HIF-1α in different types of pulp degeneration.** Immunohistochemical staining was used to evaluate HIF-1α expression and hypoxic conditions (Figs. 2 A1-D1). Positive HIF-1α expressions (black arrows) were localized in dental pulp fibrosis (Figs. 2 A1-A2), calcification (Figs. 2B1-B2), reticular atrophy (Figs. 2c1-c2), and vacuolar degeneration of the odontoblasts layer (Figs. 2D1-D2). HIF-1a is widely present in hypoxic conditions of cells. The above results confirmed that the hypoxia in the pulp of teeth with severe periodontitis might be an important cause of pulp degeneration.

Immunofluorescence staining for HIF-1a was also conducted on a representative slice of pulp degeneration.

Table I. A series of pathological changes were observed in the teeth affected by severe periodontitis.

Histopathological	Group I	Group II
Nomal	8 (10.81 %)	9 (90.00
Hyperemia	1 (1.355 %)	1 (10.00
Degeneration	22	0
Inflammation	13	0
Necrosis	30	0
Total	74	10

Significant red fluorescence was abundant, indicating positive expression of HIF-1α (Fig. 3).

Elevated expression of HIF-1a in pulp degeneration in teeth with severe periodontitis. Few positive expressions of HIF-1α were observed in normal dental pulps (Fig. 4a). Many HIF-1α-positive cells were observed in pulp degeneration (Fig. 4b). Western blotting results revealed increased expression of HIF-1α in pulp undergoing degeneration due to severe periodontitis, in comparison to normal pulp (Fig. 4c). Increased expression of HIF-1α has been observed during pulp degeneration in teeth affected by severe periodontitis.

DISCUSSION

Various potential channels, such as apical foramen, dentinal tubules, lateral and accessory root canals, exist between periodontal and dental pulp tissues, facilitating the interaction of infectious microorganisms (Vaziri *et al*., 2023). These channels may serve as pathways for the exchange of inflammatory substances between the two tissues (Samir *et al*., 2023). The pathogenic bacteria originating from periodontal or endodontic infections spread between periodontal and pulpal tissues, eventually leading to the development of combined lesions (Arulmari *et al*., 2023). Extracted teeth suffered from severe periodontitis were useful to assess dental pulp status. Early histological investigations have indicated that minimal changes are observed in pulp tissues in the majority of cases associated with moderate and advanced periodontitis (Ricucci *et al*., 2021). Periodontitis can aggravate pulp degeneration by promoting tissue injury and the release of a variety of nonspecific inflammatory mediators, including serotonin, bradykinin, histamine, arachidonic acid metabolites (prostaglandin E2), and various interleukins (Gautam *et al*., 2017).

According to our results, pulp degeneration, inflammation or necrosis are the primary forms of dental pulp tissue changes caused by periodontitis. Till now,

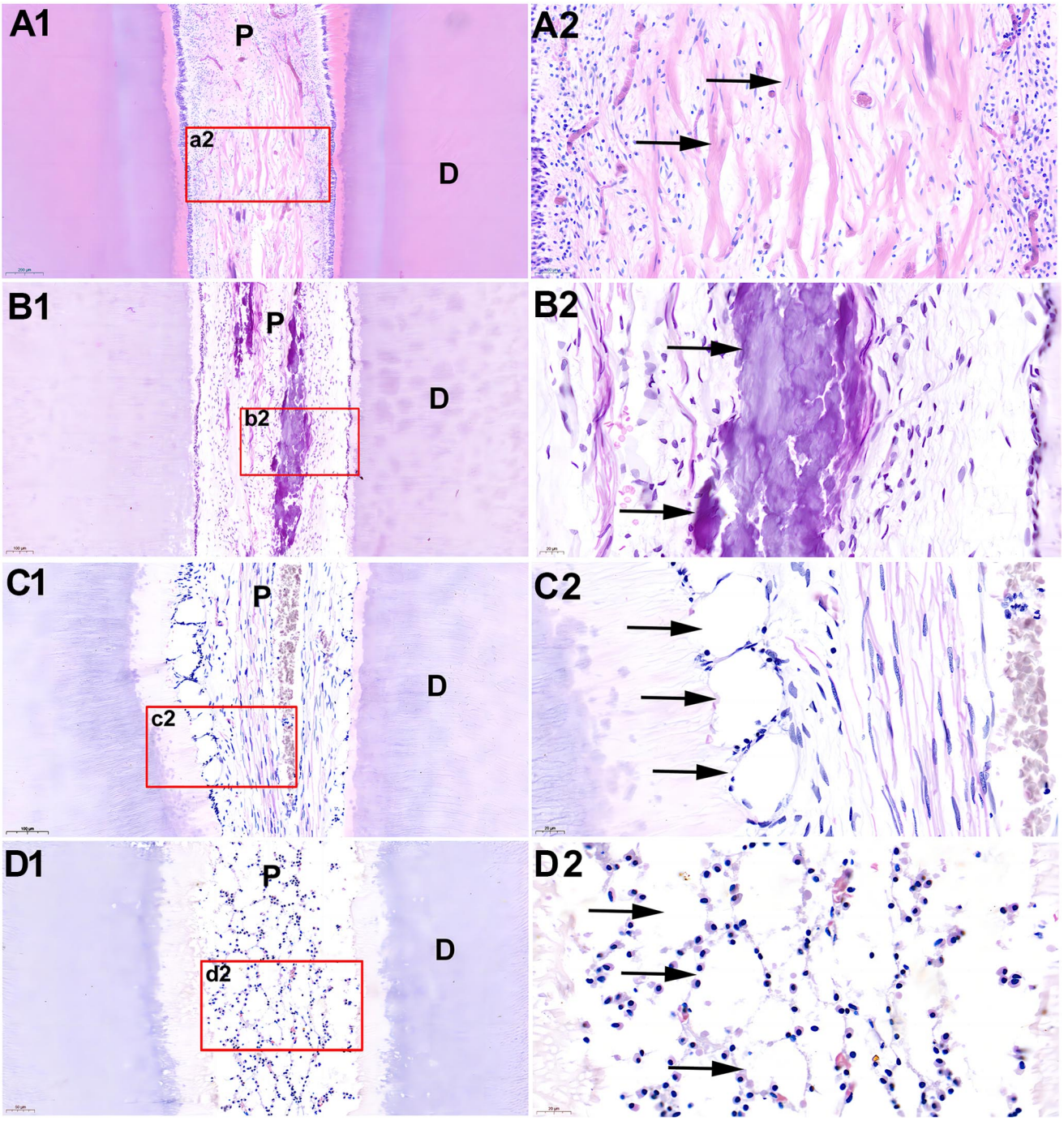

Fig. 1. Representative images of hematoxylin and eosin (HE) staining depicting pulp degeneration in teeth severely affected by periodontitis. Pulp fibrosis (a1-a2); Pulp calcification (B1-B2); Vacuolar degeneration of the odontoblastic layer (C1-C2); Reticular atrophy of the pulp (D1-D2). Scale bars=100 μm (A1-D1). Scale bars=20 μm (A2-D2). D dentin, P pulp.

research on the correlation between periodontitis and pulp degeneration remains relatively scarce, especially its related mechanisms. Local damage to periodontal microcirculation due to periodontitis can lead to remarkable hypoxia in the periodontal tissues (Yu *et al*., 2015). The expression of HIF-1α has been investigated *in vivo* and has been widely observed in tissues of animals subjected to hypoxic environments (Pugh & Ratcliffe, 2003). Some studies have reported that the expression and distribution of HIF-1α had changed in early pulp inflammation, which proves that it has a regulatory role in dental pulp inflammation (Yu *et al*., 2020). Therefore, we chose

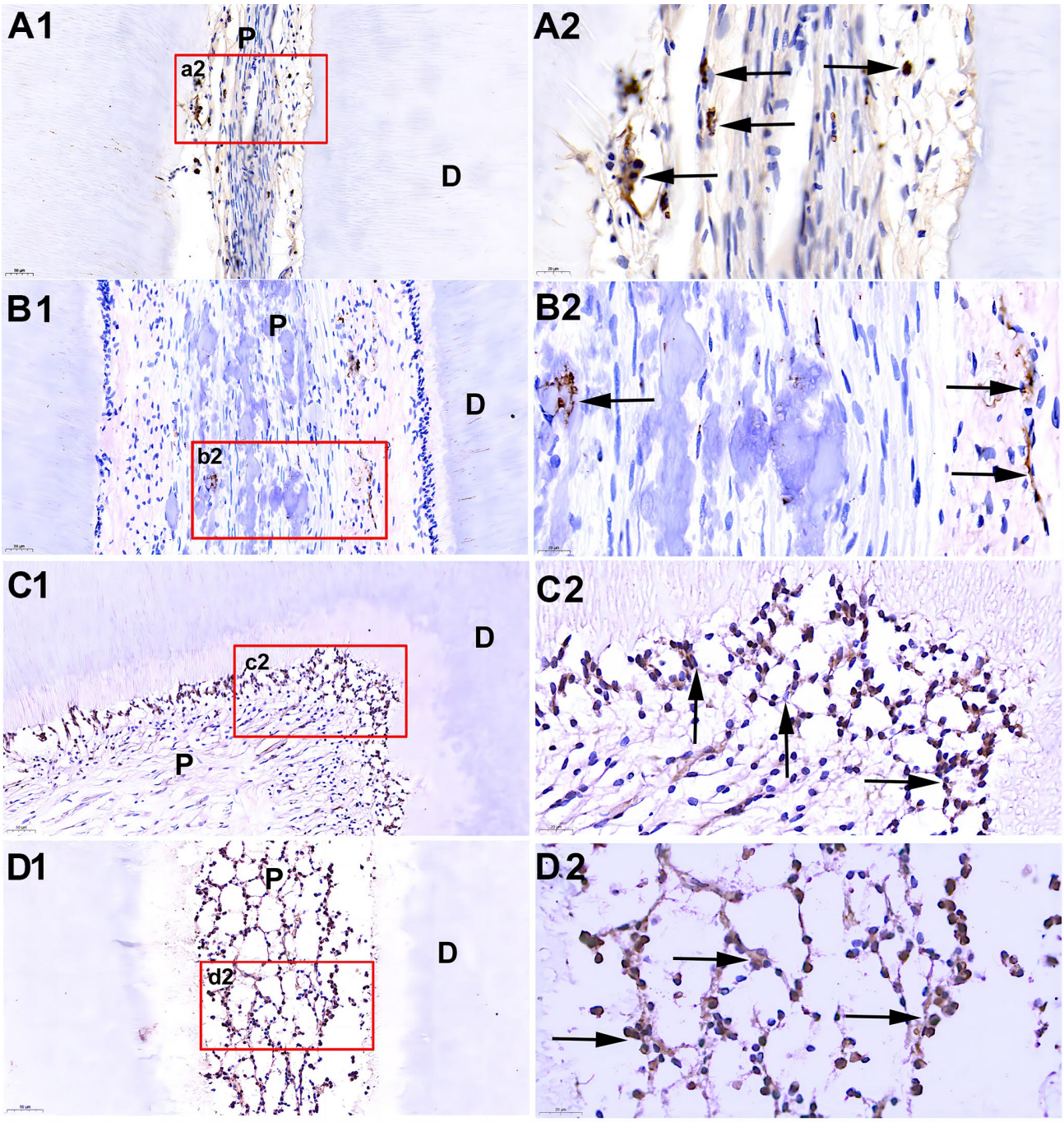

Fig. 2. Expressions of HIF-1α in the pulp of various pulp degeneration by immunohistochemistry. Positive expressions of HIF-1α (black arrows) were observed in dental pulp fibrosis (A and A2), calcification (B1 and B2), reticular atrophy(c1 and c2) and vacuolar degeneration of the odontoblasts layer (D1 and D2). Scale bars=50 μm (A1-D1). Scale bars=20 μm (A2-D2). D dentin, P pulp.

degenerative pulp to examine the expression of HIF-1α in our study. Few positive expressions of HIF-1α were observed in normal dental pulps. However, positive HIF-1α expressions were localized in typical pulp degeneration, including dental pulp fibrosis, calcification, reticular atrophy, and vacuolar degeneration of the odontoblasts layer. Western blotting results also revealed increased expression of HIF-1α in pulp undergoing degeneration due to severe periodontitis in comparison to normal pulp. Increased pulp degeneration and HIF-1α expression were observed during pulp degeneration in teeth affected by severe periodontitis.

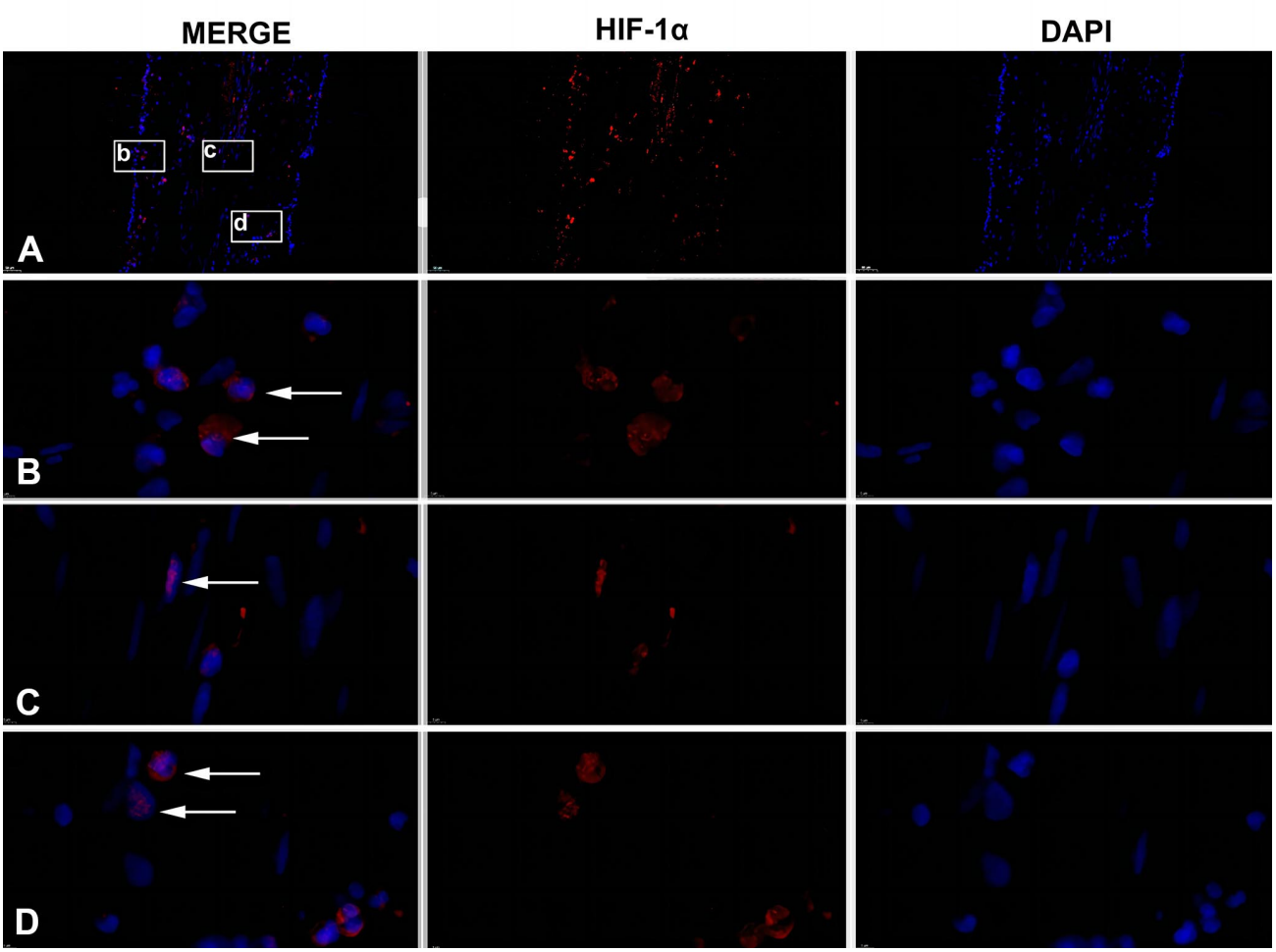

Fig. 3. Positive expressions of HIF-1α was observed by immunofluorescence. Immunofuorescence results showed that positive expressions of HIF-1α (white arrows) were observed in in the dental pulps of teeth affected by severe periodontitis (a). Higher magnifcation (b-d). Scale bars=5 μm.

Hypoxia refers to a state where tissues and cells in the body lack an adequate supply of oxygen (Semenza, 2007). Toxic products produced by microorganisms in periodontal lesions infiltrate dental pulp, favoring the proliferation of anaerobic bacteria (Li *et al*., 2019; Arias *et al*., 2023). In the cellular response to hypoxia, HIF-1α assumes a pivotal role (Cramer *et al*., 2003). Regulation of HIF-1α activity is expected to become a novel strategy for the treatment of pulp degeneration. Drugs targeting HIF-1α stabilization or transcriptional activity can alleviate hypoxia-induced tissue damage, promote pulp tissue regeneration or halt the further development of the lesion.

The effect of periodontitis on dental pulp tissue is multifaceted. This study elucidates the impact of periodontitis on the pulp degeneration and provides new ideas for the treatment of teeth affected by severe periodontitis. However, dental pulp degeneration might arise from a variety of factors, including trauma, infection, inflammation, ischemia, and other factors (Dahlén, 2002). Further investigation is required to elucidate the potential impacts of periodontitis on dental pulp degeneration.

CONCLUSION

Enhanced expression of HIF-1α in the teeth affected by severe periodontitis compared to the normal pulp. The dental pulp of teeth affected by periodontitis is more prone to pulp degeneration, with HIF-α being involved in this process.

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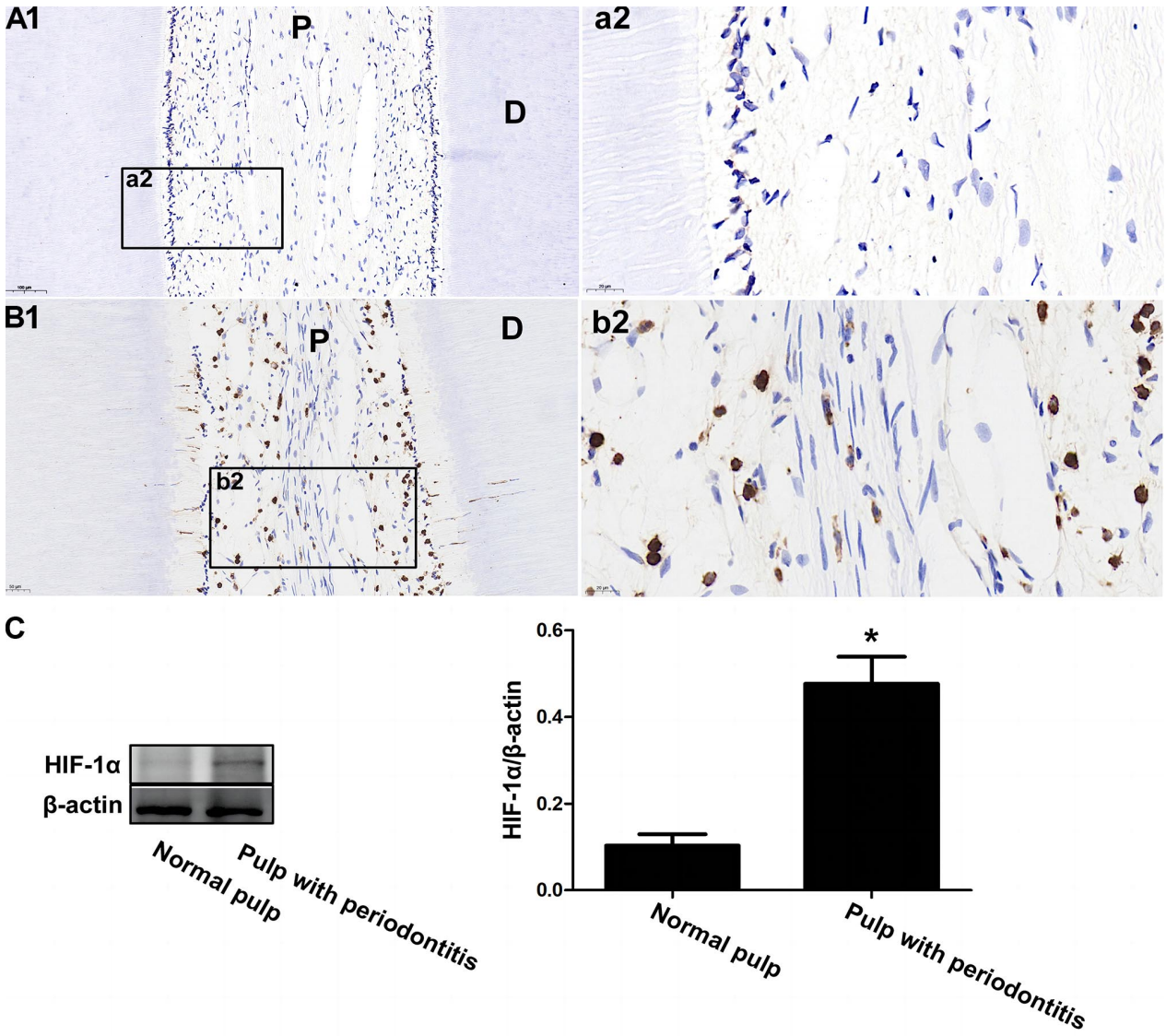

Fig. 4. Elevated expression of HIF-1a in pulp degeneration in teeth with severe periodontitis. Few positive expressions of HIF-1α (black arrows) were observed in normal dental pulps (A). Many HIF-1α-positive cells were observed in pulp degeneration (B). Compared to normal pulp, pulp undergoing degeneration due to severe periodontitis exhibited increased expression of HIF-1α, as evidenced by western blotting (C) (*p<0.05). Scale bars=50 μm (A1 and B1). Scale bars=20 μm (A2 and B2).

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RESUMEN: Aún son inconclusos los estudios sobre los efectos a largo plazo de la periodontitis crónica en la degeneración pulpar. Se seleccionaron 74 dientes extraídos de pacientes con periodontitis grave que permitiesen proporcionar información para la evaluación del estado de la pulpa dental. Se prepararon cortes de tejido y se tiñeron con HE para observar los cambios patológicos en las pulpas. Se seleccionaron lesiones típicas de degeneración pulpar para observar la expresión de HIF-1α mediante inmunohistoquímica, inmunofluorescencia y transferencia Western. No se observó degeneración pulpar en los dientes controles normales. Entre los 74 dientes analizados, 22 de ellos (30 %) presentaban degeneración pulpar con periodontitis grave. Se observó una expresión positiva de HIF-1α en odontoblastos, degeneración de células pulpares y proliferación de fibroblastos. La pulpa dental normal mostró una expresión positiva mínima. El análisis de transferencia Western reveló una elevación significativa en la expresión de HIF-1α en dientes afectados por periodontitis severa en comparación con el grupo normal. La pulpa dental de los dientes afectados por periodontitis es más propensa a la degeneración pulpar, y el HIF-1α está involucrado en este proceso.

PALABRAS CLAVE: Periodontitis; Degeneración pulpar; HIF-1α; Enfermedad combinada periodontal-endodóntica; Calcificación.

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