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Original Article

Elevated expression of importin 8 in inflamed human dental pulps

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Abstract: This study was to elucidate the roles of importin 8 in human dental pulp inflammatory activity by quantifying importin 8 expression in healthy and inflamed human dental pulps. 30 human dental pulps suffering from irreversible pulpitis caused by carious were involved in the study. The expression of IPO8 was detected by immunohistochemistry, immunofluorescence, Real-time PCR and Western blot respectively. NF-κB p p65 and p IκBα expressions in nuclear protein of dental pulps were detected to investigate NF-κB activation. Our results showed that IPO8 expression in inflamed human dental pulps increased significantly at both mRNA and protein levels compared with healthy pulps. In healthy pulps, the positive expression of IPO8 was mainly in odontoblasts. Under inflammatory conditions, IPO8 staining was also observed in nerve fiber tissues and dilated blood vessels, besides odontoblasts. NF-κB 65 and IκBα phosphorylation in cell nuclei was significantly upregulated in inflamed human dental pulps. In conclusion, IPO8 may be a marker reflecting human dental pulp inflammation. NF-κB is activated in human pulp inflammatory activity.

Keywords: IPO8, pulpitis, NF-κB

Introduction

Dental pulp inflammation (pulpitis) is a major oral health issues caused by oral bacterial infection [1]. Dental pulp inflammatory activity is a complicated process which involves tertiary dentin formation, dilated blood vessels, thickening nerve fibers and abundant inflammatory cells [2-5]. During the process, cell signals control pulp-dentin vitality and prognosis, offer us more novel and effective therapeutic strategies.

Importin 8 (IPO8), a member of the protein import receptor importin β family (also named the karyopherin β), plays a critical role in mediating the cytoplasm-to-nucleus transport in mammalian cells [6-11]. Researches show that IPO8 may mediate the translocation of NF-κB/p65 into the nucleus [12]. However, it has been reported that NF-κB maybe an important transcription factor, it may play an initial role in the inflammatory reaction [13, 14]. NF-κB unit p65 dissociates from its inhibitory protein IκB-α and translocates to the nucleus where it is activated and regulates the transcription of inflammatory mediators [13, 15]. In this study, NF-κB phosphorylation was detected to investigate NF-NF-κB/p65κB activation in inflammatory human dental pulps.

To our knowledge, there is no report on IPO8 gene expression and function in human dental pulp inflammation. It is essential to elucidate how inflammation stimulus alters IPO8 expression or how the IPO8 contributes to pulp inflammation. The present study is the first to demonstrate the expression of IPO8 in human healthy and inflamed dental pulp tissues.

Materials and methods

Study participants and sample collection

All studies were approved by the Institutional Review Board of Jinan Stomatological Hospital,

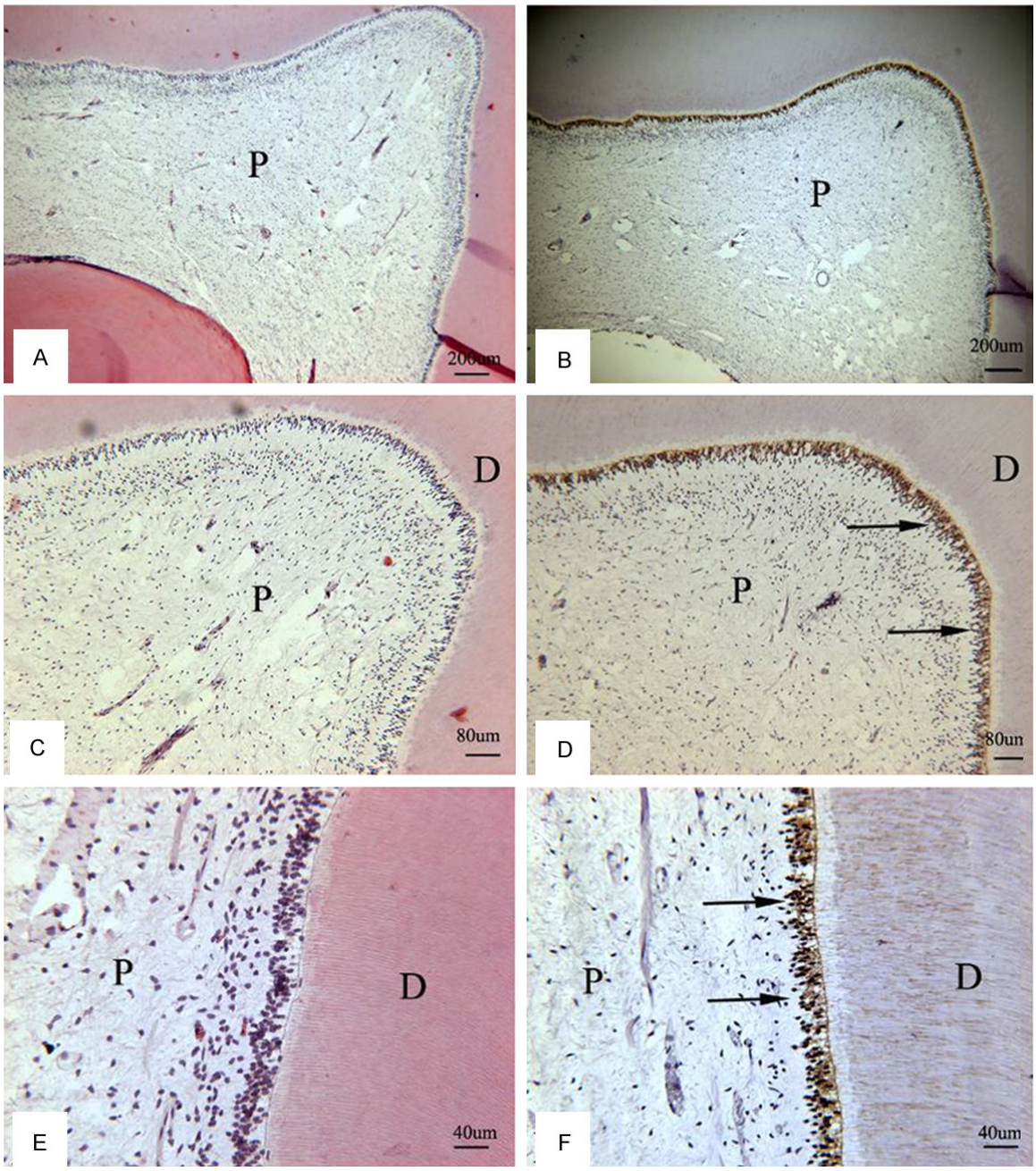


Figure 1. Histological observation and IP08 immunoreactivity in healthy human pulp. IP08 expression was found primarily in the odontoblast layer (black arrows). P (pulp), D (dentin).

and all patients provided voluntary informed consent to participate in the study. Participants (32 males and 28 females, aged 25-35years, mean 29 years) were enrolled into 2 groups on the basis of the pulpal status of the teeth being treated. Normal pulps (n=30) were extirpated from healthy third molars as control. Inflamed pulps (n=30) were extirpated from carious third molars diagnosed with irreversible pulpitis defined as both carious pulpal exposure and the presence of spontaneous pain and an exaggerated and lingering response to cold. The participants had no clinically significant medical history or any long-term anti-inflammatory medication.

The molars of each group were divided into 2 groups (10 for tissue staining, the other 20 for

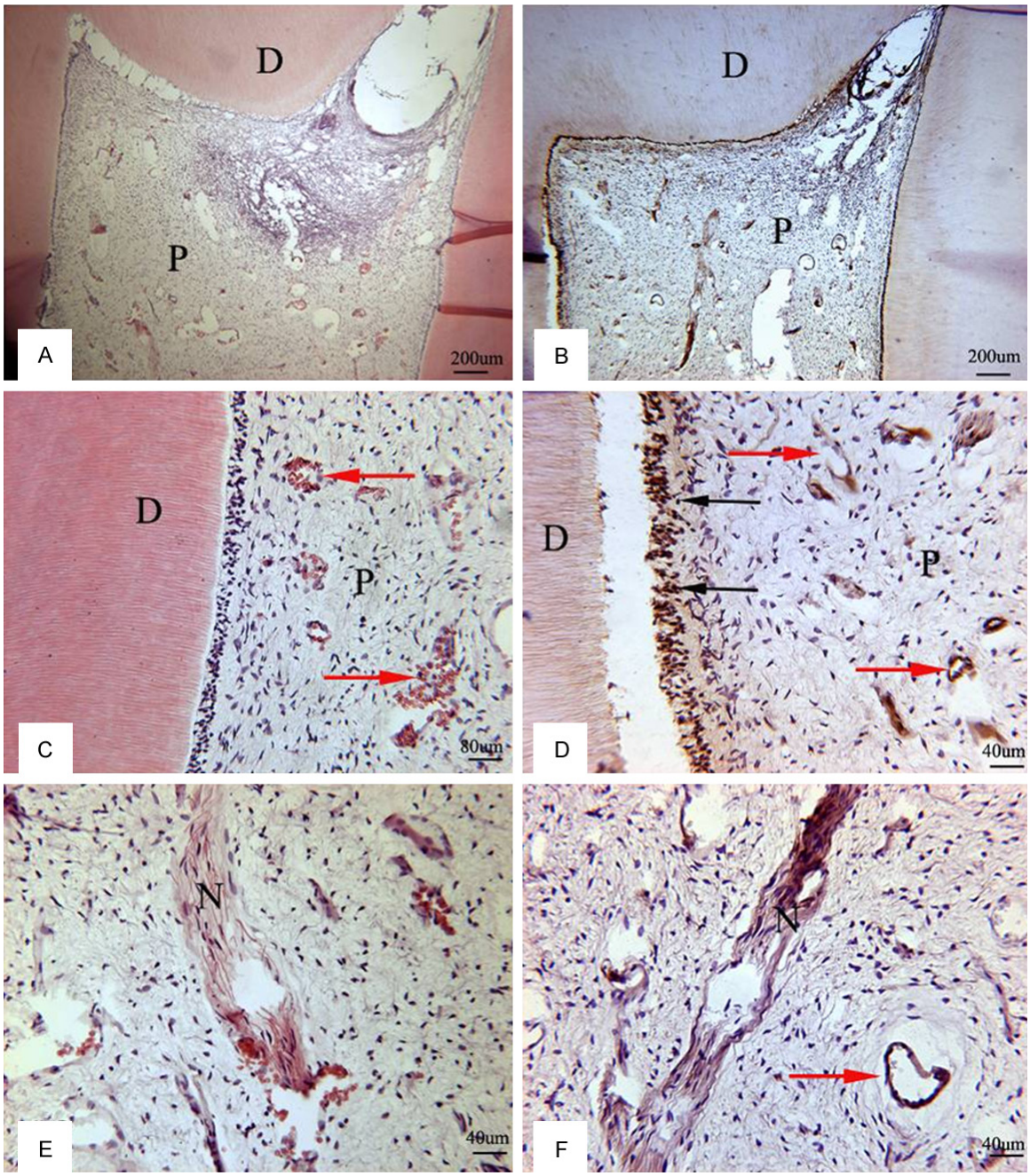


Figure 2. Histological observation and IP08 immunoreactivity in human molar pulpitis. IP08 expression was found primarily in odontoblasts layer (black arrows), blood vessels (red arrows) and thickening nerve fiber. P (pulp), D (dentin), N (nerve).

molecular study) randomly. The teeth for tissue staining were fixed with 4% paraformaldehyde (PFA) for 24 hours, followed by demineralization for 8 months in 12.5% EDTA. Serial sections of 5 um in thickness were made for H.E, immunohistochemistry and immunofluorescence. The teeth for real-time PCR and western blots study were not fixed, and the fresh pulp tissues were acquired from the extracted tooth and then rapidly frozen in liquid nitrogen.

Immunohistochemistry

Immunohistochemical study was performed by using Streptavidin-Peroxidase kit (Zhongshan, Beijing, China). Sections were deparaffinized

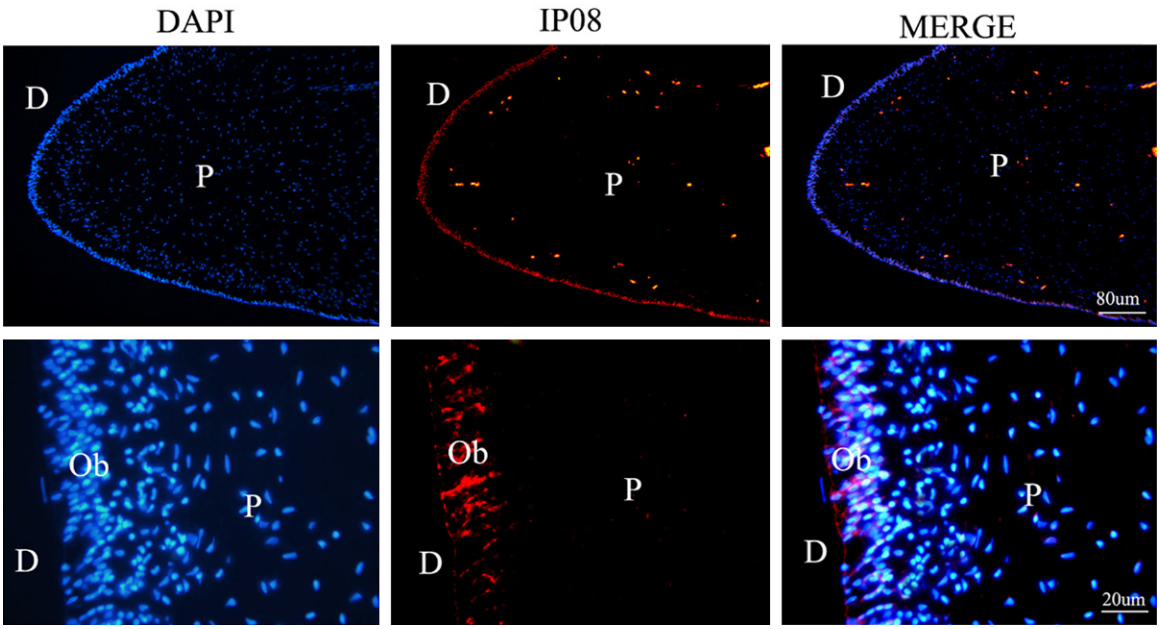


Figure 3. In healthy pulps, IP08 positive expression is detected in the cytosol of the odontoblast bodies. D (dentin), Odontoblasts layer (Ob). P (pulp).

and rehydrated in a series alcohol, and then the sections were treated with 3% H_2O_2 to block endogenous peroxidase activities as previously described [16], and reacted with polyclonal antibody IP08 (dilution 1:50, Abcam, UK) overnight at 4°C. PBS was obtained as negative control. After washing with PBS (pH 7.4), the sections were incubated with a biotinylated goat anti-rabbit immunoglobulin G followed by incubation with conjugated streptavidin-peroxidase (Zhongshan, China) as previously described [16], Diaminobenzidine (DAB) solution (Zhongshan, China) was used to visualize localization for 2 min. Finally, Nuclei were counterstained with hematoxylin solution (Zhongshan, China) for 2 min. The sections were examined and photographed with a light microscope (OLYMPUS CX-71, Japan).

Immunofluorescence

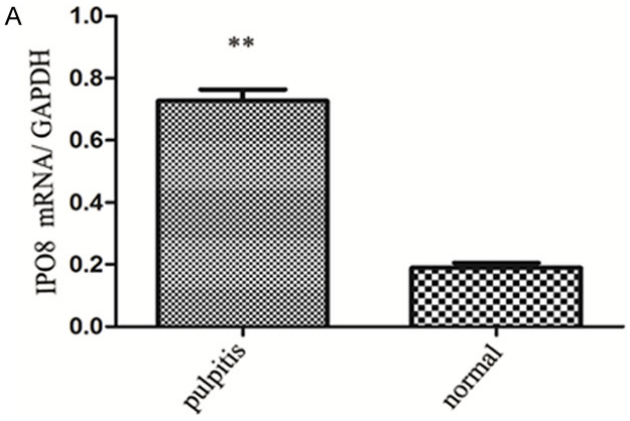
Sections were deparaffinized in xylene and rehydrated. After washing, the sections were reacted with polyclonal antibody IP08 (dilution 1:50, Abcam, UK) at 4°C overnight. The sections were then incubated with rhodamine (TRITC)-conjugated goat anti-rabbit IgG (Sigma, USA) for 1 h at room temperature. Nuclei were stained with DAPI solution (Sigma, USA) for 5 min. The sections were photographed with immunofluorescence microscopy (OLYMPUS BX-60, Japan).

RNA extraction and quantitative real-time PCR

Samples (10 Healthy samples, 10 pulpitis samples) were processed for RNA extraction using TRIzol reagent (TaKaRa, Japan). The purity and concentrations of the RNA samples were measured using Gene Quant Pro UV/visible spectrophotometer (Biochrom Ltd, UK). Then 1 μg total RNA was reverse-transcribed into cDNA by a cDNA synthesis kit (TaKaRa, Japan) according to the directions. Thereafter, Real-time PCR was performed with SYBR Green I (TaKaRa, Japan) and LightCycler 480 Real-Time PCR instruments (Roche, Germany). GAPDH was used as internal standard control, and the sequences of the primers as follows: GAPDH sense 5'- GCACCGTCAAGGCTGAGAAC-3', GAPDH antisense 5'- TGGTGAAGACGCCAG-TGGA-3'; IP08 sense 5'-GGACCGTTCCTCCTG-AGACT-3', IP08 antisense 5'-TGTGACATTTCC-TGGGCTTC-3'.

Protein extraction and Western blots

20 samples (10 Healthy samples, 10 pulpitis samples) were washed by cold PBS three times respectively, and then homogenized in RIPA buffer for 30 min. 1 mM phenylmethane sulfo-



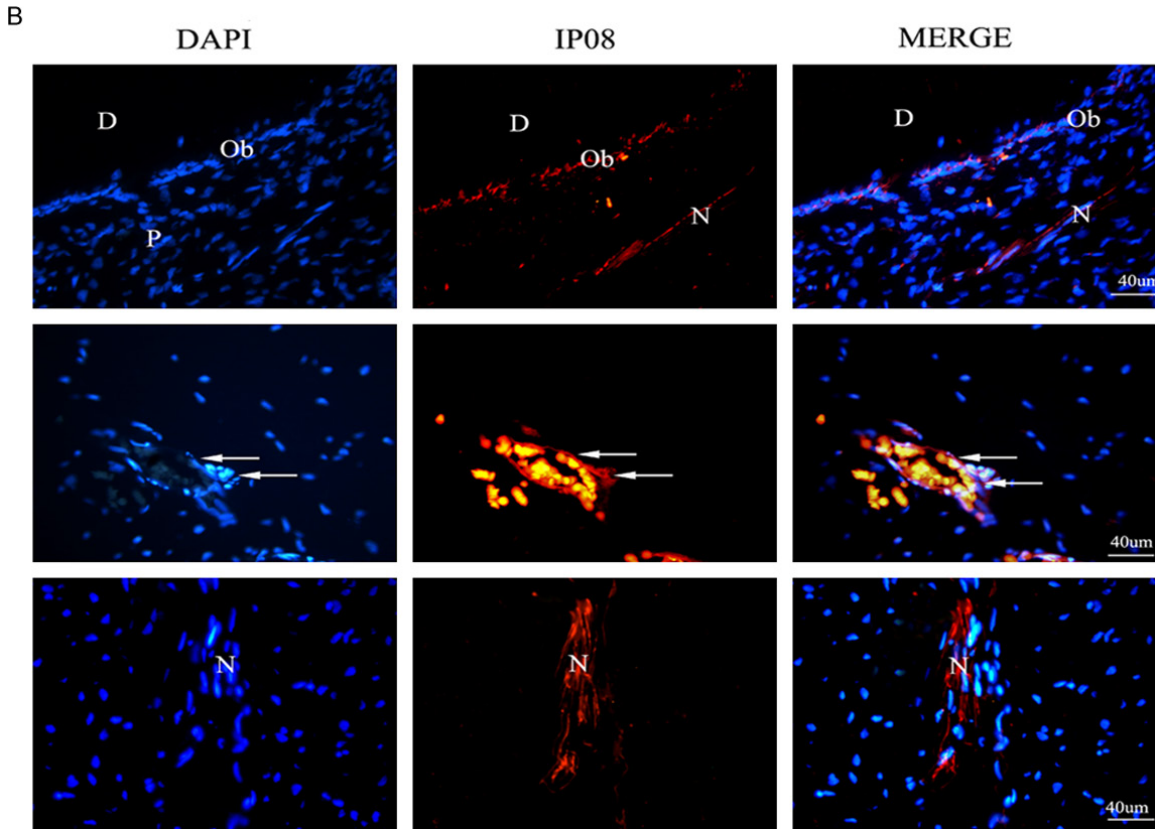


Figure 4. A. Expressions of IPO8 mRNA in human healthy and inflamed dental pulps. IPO8 mRNA was increased in the inflammatory pulp tissues (**$P < 0.01$). B. IPO8 immunofluorescence in human pulpitis. IPO8 expression was detected in odontoblasts, blood vessels (white arrows) and nerve fiber tissues. Odontoblasts (Ob). D (dentin), P (pulp), N (nerve fiber).

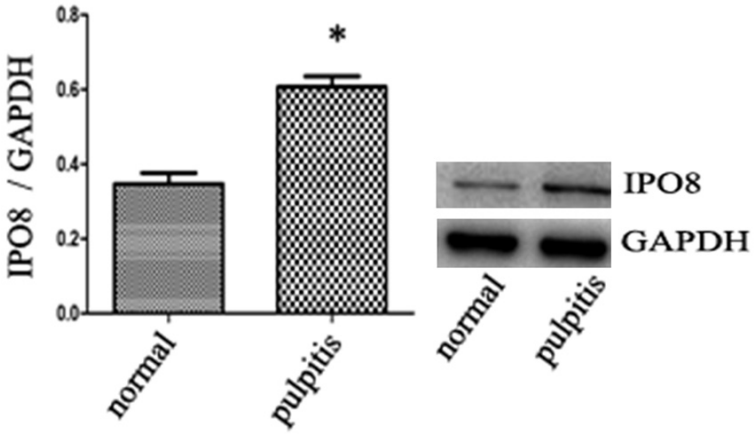


Figure 5. Expressions of IPO8 protein in human healthy and inflamed dental pulps. Higher expression of IPO8 protein was detected in the inflammatory dental pulp, comparing with human healthy dental pulps (*$P < 0.05$).

nyl-fluoride was added into the buffer in advance. The nuclear fraction of samples was extracted using The Ambion® PARIS™ system (Thermo Fisher). Protein concentra-

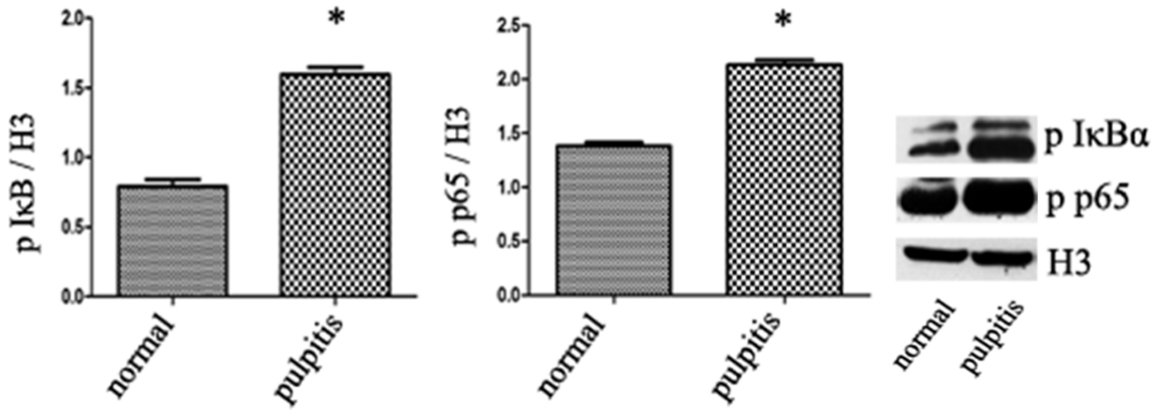


Figure 6. Expressions of NF-κB p p65 and p IκBα in human healthy and inflamed dental pulps. Elevated expressions of NF-κB p p65 and p IκBα were detected in cell nuclei protein of inflamed dental pulps.

tions were measured using a bicinchoninic acid assay (BCA) protein quantitative analysis assay kit (BOSHE, China); Proteins were separated on 10% SDS gels and then transferred onto polyvinylidene difluoride membranes (Millipore, USA). After blocked in 0.1% Tween 20 in Tris-buffered saline (TBST) containing 5% nonfat dried milk for 1 h at room temperature, the membranes were incubated with the IPO8 antibody (1:500), NF-κB p p65 (1:500) and p IκBα (1:500) overnight at 4°C. Before incubation with horseradish peroxidase (HRP)-labeled second antibody (Beyotime), the membranes were rinsed with TBST 10 min×3 times. The bands were visible on the Canon films using ECL substrate solution (Millipore). GAPDH (1:10000) or H3 was used as internal control.

Statistical analysis

All of the images of the real-time PCR and western blots assays were representative of at least three independent experiments. All measurement results were presented as the mean ± SD. All values were calculated using Student's t-test. The differences were considered to be statistically significant at $P < 0.05$.

Results

Immunohistochemistry and immunofluorescence

Healthy dental pulp tissues were composed of loose connective tissues including fibroblasts, nerve fiber tissues, blood vessels, and the surrounding odontoblastic layer (**Figure 1A**, **1C**, **1E**). IPO8 positive expression was primarily localized in the odontoblasts (**Figure 1B**, **1D**, **1F**).

In the pulpitis, a diffuse, strong pulpal and perivascular lymphocytic infiltration was detected. Numerous blood vessels were filled with erythrocytes (**Figure 2A**, **2C**, **2E**), IPO8 positive staining was expressed in numerous blood vessels and thick nerve fiber tissues, which were not stained in healthy conditions (**Figure 2B**, **2D**, **2F**).

In the inflamed dentin-pulp complex, IPO8 positive staining was expressed in numerous blood vessels and thickening nerve fiber tissues (**Figure 4B**), which were not stained or absent in healthy conditions (**Figure 3**).

IPO8 mRNA level by real-time PCR analysis

The analysis of IPO8 mRNA in human healthy and inflamed dental pulps was detected by Real-time PCR. Higher expression of IPO8 mRNA was detected in the inflammatory pulp tissues, comparing with human healthy dental pulps (*$P < 0.01$) (**Figure 4A**).

IPO8 protein level by western blots analysis

IPO8 protein could be detected in human healthy dental pulps, but the expression of IPO8 protein was significantly up-regulated in the inflammatory pulp tissues (*$P < 0.05$) (**Figure 5**).

There was higher expression of NF-κB p p65 and p IκBα in cell nuclei protein of inflamed dental pulps than those in human healthy den-

tal pulps. The result showed NF-κB 65 and IκBα phosphorylation in cell nuclei was significantly upregulated. NF-κB is activated in human pulp inflammatory activity (**Figure 6**).

Discussion

To our knowledge, Odontoblast layer is the first barrier during the inflammation defense and form secondary dentin at a relative slower speed in normal mature tooth [17-19]. The results showed that IP08 expression was found in the odontoblast layer in both healthy and inflammatory pulps. Its expression indicates that IP08 might play an important role in odontoblast differentiation, and may participate in the formation of dentin whether in normal and in inflammatory situation.

But in the inflammatory pulps IP08 was also detected in thickening nerve fiber and vascular endothelial cells filled with erythrocytes, which represented the dental pulp inflammatory activity. Increased IP08 expression in nerve fibers of inflammatory pulp tissues indicated that mechanism of dental pulp inflammation might include a neuroregulation component mediated by IP08. Elevated expression of IP08 in vascular endothelial cells contributed to the disruption of the barrier of endothelial cells, thereby increasing the vascular permeability and inducing tissue edema, which in turn produced the elevation of pressure in pulpal cavity and induced more severe inflammation responses. Thus, IP08 might be a novel target for anti-inflammatory therapy through the pathway of vasopermeability mediation. Results of real-time PCR and Western blots further verified higher expression of IP08 in inflammatory dental pulps, which indicated IP08 participated in dental pulp inflammatory responses.

NF-κB, an important transcription factor, plays critical roles in inflammatory responses [13, 14, 18, 20, 21]. A survey of the literature available shows that IP08 may mediate the translocation of NF-κB/p65 into the nucleus where it is activated and regulates the transcription of inflammatory mediators [12, 22]. Our results showed NF-κB 65 and IκBα phosphorylation in cell nuclei was significantly upregulated. NF-κB is activated in human pulp inflammatory activity. During the process of dental pulp inflammation, whether NF-κB participated in the inflammation process was mediated by interacting with IP08 or whether the interaction was regulated by NF-κB pathway awaits further investigation.

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Disclosure of conflict of interest

None.

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