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ORIGINAL ARTICLE

Observation of Inflammation, Oxidative Stress, Mitochondrial Dynamics, and Apoptosis in Dental Pulp Following a Diagnosis of Irreversible Pulpitis

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ABSTRACT

Objective: Mitochondrial dynamics play a pivotal role in maintaining the homeostasis of the dental pulp. Inflammation and oxidative stress can trigger changes in mitochondrial dynamics, leading to cell death in the dental pulp. This study aimed to investigate inflammation, oxidative stress, mitochondrial dynamic alterations, and cell death in inflamed pulpal tissues compared to healthy pulp tissues.

Methods: Pulpal tissues were collected (n=15 per group) from: 1) healthy people as the control and 2) people with clinically diagnosed irreversible pulpitis. Proteins indicating inflammation, oxidative stress, mitochondrial dynamics, and cell death markers were investigated by western blot analysis. A Student's t-test was used to analyse differences between the healthy and irreversible pulpitis groups. A probability of 0.05 was used to indicate statistical significance ($p<0.05$).

Results: The expression of the proteins, tumour necrosis factor-alpha (TNF-α) and nuclear factor kappa-light-chain-enhancer, by activated B cells (NF-κB) from inflamed pulp tissues were significantly higher than those of control. Compared to controls, 4 hydroxynonenal (4HNE) and dynamin-related protein 1 (Drp1) were significantly higher, while mitofusin 2 (MFN2) and optic atrophy type 1 (OPA1) were significantly lower in inflamed pulp tissues. Bcl-2-associated X protein (Bax), cleaved caspase-3, and cytochrome c were significantly higher in inflamed pulpal tissues compared to controls. In inflamed pulpal tissues, we found a significant increase in the expression of receptor-interacting serine or threonine-protein kinase 1 (RIPK1) but not receptor-interacting serine or threonine-protein kinase 3 (RIPK3).

Conclusion: Irreversible pulpitis is associated with inflammation, oxidative stress, alterations in mitochondrial dynamics, and apoptosis in pulpal tissues.

Keywords: Mitochondrial fission, mitochondrial fusion, pulp inflammation

HIGHLIGHTS

- Irreversible pulpitis exhibits an increase in inflammation and oxidative stress
- An imbalance in mitochondrial dynamics associated with irreversible pulpitis
- Apoptosis but not necroptosis is involved in irreversible pulpitis.

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INTRODUCTION

It has been reported that thirty-four per cent of the world's population have untreated dental caries (1). Dental caries is reputably considered one of the most widespread diseases in the global population and is primarily responsible for inflammation of the dental pulp (1). Therefore, seeking novel findings for the restoration

of caries-induced pulpitis would benefit society. One way of achieving an effective approach to treating pulpitis is to understand better the molecular biology aspects of pulpitis at a cellular level. Studies have shown that the pathophysiology of dental caries-induced pulpitis includes increased production of inflammatory cytokines, oxidative stress, damaged mitochondria, and cell death (2–4). In the physiological condition, mitochondria are important for sustaining the homeostasis of the dental pulp (5, 6). A disturbance in the balance of mitochondrial dynamics, including mitochondrial fusion and fission, triggers dyshomeostasis in the dental pulp tissue, resulting in cellular oxidative stress and cell death (2, 5).

It has been widely reported that disturbance in the balance of mitochondrial dynamics can have an impact on cellular functions (7, 8). There is extensive research to demonstrate that inflammation is one of the key processes in the instigation of changes in mitochondrial dynamics (2, 9, 10). Our previous studies demonstrated that the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling pathway can alter mitochondrial dynamics in the heart and brain (10–12). However, to date, only Yang et al. (2) reported that tumour necrosis factor-alpha (TNF-α) contributes to changes in mitochondrial dynamics in inflamed human dental pulp tissues.

There have been a few studies into changes in mitochondrial dynamics and inflammation in the human dental pulp. Oxidative stress has been identified as one of the factors that can disrupt mitochondrial dynamics (11, 13). Our previous study demonstrated that oxidative stress had been associated with changes in mitochondrial dynamics in cardiac tissues (11). Only a few studies have shown that oxidative stress can change mitochondrial dynamics in mouse pre-odontoblasts (2, 14). There has been limited research into the association between oxidative stress and the disturbance of mitochondrial dynamics in the human dental pulp.

Numerous studies have reported that inflammation and oxidative stress cause dental pulp cell death (3, 15). Wang et al. (3) demonstrated the expression of an apoptotic marker in inflamed human dental pulp and the occurrence of apoptosis in lipopolysaccharide (LPS)-treated mouse pre-odontoblastic cells. Hydrogen peroxide-induced apoptosis was also observed in isolated pulpal cells from humans and rats (15, 16).

Necroptosis is one of the forms of cell death that can be activated during inflammation and is primarily induced by TNF-α (17, 18). However, only a limited number of studies have focused on the occurrence of necroptosis in clinical pulpitis (19). Therefore, this study aims to determine the levels of inflammation, oxidative stress, mitochondrial antioxidants, mitochondrial dynamics, apoptosis, and necroptosis in human inflamed pulp tissues compared to normal healthy pulp tissues. We hypothesised that an increase in inflammatory cytokines, alterations in oxidative stress and mitochondrial antioxidants, disturbance of mitochondrial dynamics, and an increase in apoptosis and necroptosis are associated with irreversible pulpitis. The null hypothesis is that there is no difference in the level of inflammatory cytokines, oxidative stress, mitochondrial antioxidants, mitochondrial dynamics, and cell death between healthy pulp and tissue with irreversible pulpitis.

MATERIALS AND METHODS

Selection of Subjects

This study was conducted in accordance with the Declaration of Helsinki (20). The study protocol was approved by the Human Experimentation Committee of the Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand (No: 44/2020, 22 June 2020). Samples from healthy human dental pulp (n=15) and inflamed human dental pulp (n=15) were extracted from permanent teeth of maxillary or mandibular molars from subjects aged 10–66. In the case of underage patients (<18 years old), consent was obtained from parents or caregivers. After informed consent was obtained, the healthy pulp tissues were collected from patients scheduled for tooth extraction or surgical removal for orthodontic purposes. The inflamed human dental pulp tissues were collected from patients presenting with teeth with symptomatic or asymptomatic irreversible pulpitis who had been assigned for tooth extraction due to endodontic treatment denial. The inclusion criteria for irreversible pulpitis were signs and symptoms of irreversible pulpitis and a positive response to the cold and electric pulp test. Patients presenting with systemic diseases, pulp necrosis, or periodontitis (periodontal pocket >4 mm, periodontal abscess, or severe tooth mobility) were excluded from the study. The clinical diagnosis of irreversible pulpitis was made following the guidelines of the American Association of Endodontists (AAE) (21). In this study, cases of both symptomatic and asymptomatic irreversible pulpitis were included. The clinical diagnosis of normal pulp was based on being symptom-free, typically responsive to pulp testing with mild or temporary response to cold testing (lasts ≤1–2 s after a stimulus is taken away), and the absence of dental caries or restorations or fractures. In the case of teeth diagnosed with symptomatic irreversible pulpitis, the carious teeth had presented with a history of spontaneous pain and response to thermal stimuli with sharp or lingering pain. In the case of asymptomatic irreversible pulpitis, the diagnosed teeth had clinical findings corresponding to those with symptomatic irreversible pulpitis, but clinical symptoms were absent and response to thermal stimuli was normal.

Following tooth extractions, the dental pulp was collected following the splitting of the tooth and tissues were frozen immediately at –80°C until further analysis.

Dental Pulp Tissue Collection

After tooth extraction, teeth were disinfected by immersion in 5.25% sodium hypochlorite (NaOCl) for 1 min, then rinsed with 0.9% sodium chloride solution. A low-speed handpiece and a straight carbide bur were used to create a buccal groove from the cementoenamel junction (CEJ) to the root furcation area. Next, an elevator was used to break each tooth in half. Dental pulp tissues were collected and immediately placed in liquid nitrogen for 30 s, then kept at –80 ºC until analysis. After all the samples had been obtained, inflammation, oxidative stress, antioxidant, mitochondrial dynamics, apoptosis, and

necroptosis markers were investigated. The diagram of the experimental protocol is shown in Appendix 1.

The Determination of Inflammation, Oxidative Stress, and Antioxidant Levels in Irreversible Pulpitis Tissues

The levels of TNF-α and NF-κB, as representatives of pulpal inflammation, were identified. The level of oxidative stress in irreversible pulpitis samples was examined by investigating the level of expression of 4 hydroxynonenal (4HNE). To study the level of antioxidants in irreversible pulpitis tissues, the level of expression of superoxide dismutase 2 (SOD2) was investigated.

After dental pulp tissue collection, the pulp tissues were lysed with lysis buffer and centrifuged at 14,000 g for 15 min. The protein concentration was measured using BCA (bicinchoninic acid) protein assay (Sigma-Aldrich, St. Louis, MO, USA). Then, the protein samples were loaded and relocated onto nitrocellulose membranes. After that, immunoblots were carried out, blocking occurring for an hour with either 5% nonfat dry milk in tris-buffer saline (pH 7.4) consisting of 0.1% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) (TBST) or 5% bovine serum albumin in TBST. The immunoblots were probed overnight at 4°C with the primary antibodies as follows: anti-TNF-α (Abcam, Cambridge, UK; ab9635); anti-NF-κB p65 (Cell Signaling Technology, Danvers, MA, USA; #8242S); anti-phospho-NF-κB p65 (Ser536) (Cell Signaling Technology, Danvers, MA, USA; #3033S); anti-4 HNE (Abcam, Cambridge, UK; ab46545); anti-SOD2 (Cell Signaling Technology, Danvers, MA, USA; #13194S), and anti-β-Actin (Santa Cruz Biotechnology, Dallas, TX, USA; SC-47778). All primary antibodies were prepared at a dilution of 1:1,000. Following immersion in the primary antibodies, a 1 h incubation with secondary antibodies was performed. The membranes were visualised and scanned with the ChemiDoc Touch Gel Imaging System (Bio-Rad Laboratories, Hercules, CA, USA). The western blotting images were interpreted using ImageJ 1.52a (Wayne Rasband, Bethesda, MD, USA) analysis software (22, 23).

The Expression of Mitochondrial Dynamics

To determine mitochondrial dynamics in the irreversible pulpitis tissue, the protein representing mitochondrial fission dynamin-related protein 1 (Drp1), and proteins related to mitochondrial fusion mitofusin 1 (MFN1), mitofusin 2 (MFN2), and optic atrophy type 1 (OPA1) were investigated by western blot analysis. The detailed steps for the western blot test were as mentioned in the previous section. In brief, after the protein samples were loaded and subjected to gel electrophoresis and relocated onto nitrocellulose membranes, the immunoblots were probed overnight at 4°C with the primary antibodies as follows: anti-Drp1 (Cell Signaling Technology, Danvers, MA, USA; #5391S); anti-phospho-Drp1 (Ser616) (Cell Signaling Technology, Danvers, MA, USA; #3455S); anti-MFN1 (Cell Signaling Technology, Danvers, MA, USA; #13196S); anti-MFN2 (Cell Signaling Technology, Danvers, MA, USA; #9482S); anti-OPA1 (Cell Signaling Technology, Danvers, MA, USA; #80471S), and anti-β-Actin (Santa Cruz Biotechnology, Dallas, TX, USA; SC-47778). The primary antibodies were prepared at a dilution of 1:1,000. After incubation with secondary antibodies, the membranes were visualised and scanned with the ChemiDoc Touch Gel Imaging System (Bio-Rad Laboratories, Hercules, CA, USA). ImageJ 1.52a (Wayne Rasband, Bethesda, MD, USA) analysis software was used to interpret the western blot images (22, 23).

Evaluation of Apoptosis and Necroptosis

Western blot analysis was performed to determine whether apoptosis and necroptosis were involved in irreversible pulpitis. We investigated the expression of Bcl-2-associated X protein (Bax), B-cell lymphoma 2 protein (Bcl-2), caspase-3, and cytochrome c, representing apoptosis markers. In addition, to study necroptosis, the presence and levels of receptor-interacting serine or threonine-protein kinase 1 (RIPK1) and receptor-interacting serine or threonine-protein kinase 3 (RIPK3) were investigated. In summary, after the process of the loading of the protein samples and the transfer onto nitrocellulose membranes, the immunoblots were immersed at 4°C overnight in primary antibodies as follows: anti-Bax (Cell Signaling Technology, Danvers, MA, USA; #2772S); anti-Bcl-2 (Cell Signaling Technology, Danvers, MA, USA; #2876S); anti-caspase-3 (Cell Signaling Technology, Danvers, MA, USA; #14220S); anti-cytochrome c (Cell Signaling Technology, Danvers, MA, USA; #4272S); anti-RIPK1(Cell Signaling Technology, Danvers, MA, USA; #3493S); anti-phospho-RIPK1 (Ser166) (Cell Signaling Technology, Danvers, MA, USA; #31122S); anti-RIPK3 (Cell Signaling Technology, Danvers, MA, USA; #15828S); anti-phospho-RIPK3 (Ser232) (Abcam, Cambridge, UK; ab195117), and anti-β-Actin (Santa Cruz Biotechnology, Dallas, TX, USA; SC-47778). All primary antibodies were diluted at a ratio of 1:1,000. After secondary antibody incubation, the membranes were visualised, scanned, and analysed as previously described (22, 23).

Statistical Analysis

Data were presented as the mean±standard deviation (SD). The normality of data distribution was confirmed by a Shapiro-Wilk test. In addition, an unpaired t-test was carried out to compare the results from the irreversible pulpitis to those of the healthy pulp. All statistical analysis was conducted using GraphPad Prism 8.2.1 software for Mac (Dennis Radushev, San Diego, CA, USA). A significance level of 0.05 was applied in this study ($p<0.05$).

RESULTS

The Demographic Data

In this study, samples from 15 patients with healthy pulp (5 males and 10 females; aged 15–34 years, mean 21.32 years old) and 15 samples from patients who were diagnosed with symptomatic or asymptomatic irreversible pulpitis (7 males and 8 females; aged 10–66 years, mean 37.55 years old) were included. All 30 subjects enrolled in this study met the inclusion criteria and complied with the approved protocol as previously described.

Inflammation, Oxidative Stress, and Mitochondrial Antioxidant in Inflamed Pulpal Tissues are Greater Than Those in Healthy Pulpal Tissues

Inflamed pulpal tissues showed a marked increase in TNF-α levels compared to healthy pulp tissues ($p<0.05$; Fig. 1a). In addition, the samples of inflamed pulpal tissues revealed A significant elevation of the ratio of p-NF-κB divided by the total NF-κB expression was found in the samples of inflamed pulpal tissues in comparison with the healthy pulp group

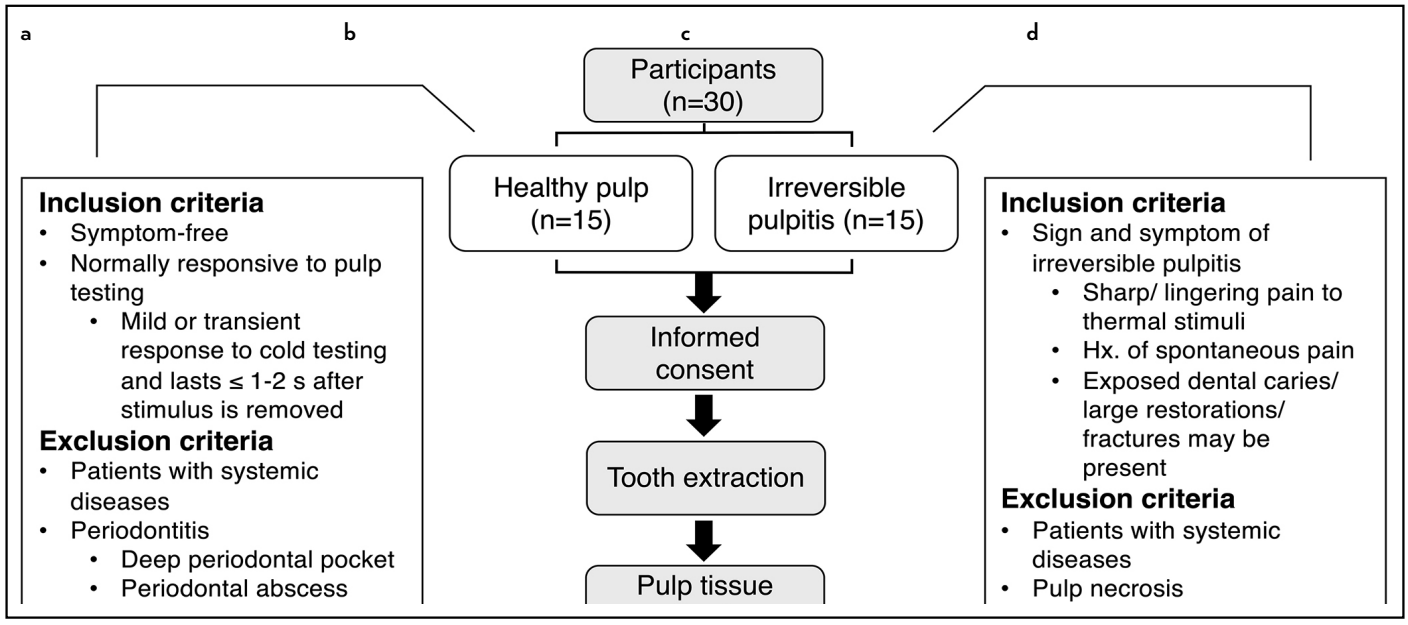

Figure 1. The characteristics of inflammatory, oxidative stress, and antioxidant profiles in irreversible pulpitis. Expression levels of TNF-α (a, e), the ratio of p- NF-κB to total NF-κB (b, f), 4HNE (c, g), and SOD2 (d, h) in healthy human pulp and irreversible pulpitis samples

Data are shown as mean±SD (n=15). *p<0.05, **p<0.01 and ***p<0.001 denotes a statistically significant difference from the healthy pulp group. H: Healthy pulp, P: Irreversible pulpitis, p-NF-κB: Phosphorylated-nuclear factor kappa-light-chain-enhancer of activated B cells, TNF-α: Tumour necrosis factor-alpha, NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells, 4HNE: 4 hydroxynonenal, SOD2: Superoxide dismutase 2

(p<0.05; Fig. 1b). These findings suggested that the inflammatory condition has occurred via the NF-κB signalling pathway in cases of irreversible pulpitis.

The expression of 4HNE, a marker of oxidative stress, was considerably higher in irreversible pulpitis tissues than those from healthy pulpal participants (p<0.05; Fig. 1c). In addition, the mitochondrial antioxidant SOD2 was also greater in inflamed pulpal tissues than in healthy pulpal tissues (p<0.05; Fig. 1d).

An Imbalance in Mitochondrial Dynamics was Observed in Irreversible Pulpitis

The ratio of p-Drp1 to Drp1 was higher in inflamed pulpal tissues than in healthy pulp (p< 0.05; Fig. 2a). Irreversible pulpitis revealed no change in MFN1 expression level (p>0.05; Fig. 2b). However, significantly lower levels of MFN2 (p<0.05; Fig. 2c) and OPA1 (p<0.05; (Fig. 2d) were observed when compared to the healthy pulp group.

Apoptosis, But not Necroptosis, was Seen in Irreversible Pulpitis

The expressions of the ratio of Bax to Bcl-2 (Fig. 3a), cleaved caspase-3 (Fig. 3b), and cytochrome c (Fig. 3c) were higher in inflamed pulpal tissues than in healthy pulpal tissue (p<0.05). In addition, the expression ratio of p-RIPK1 divided by total RIPK1 was significantly greater in irreversible pulpitis than in healthy pulp (p<0.05; Fig. 3d). However, the ratio of the expression of the p-RIPK1 to total RIPK1 did not differ between the healthy and inflamed pulp tissues (p>0.05; Fig. 3e).

DISCUSSION

The main findings of this study are: 1) irreversible pulpitis tissue exhibited inflammation; 2) oxidative stress and mitochondrial antioxidants were found to be higher in inflamed pulpal tissues in comparison with healthy pulpal tissue; 3) irreversible pulpitis tissue showed a disturbance in the balance of mitochondrial dynamics, as demonstrated by increased mitochondrial fission and reduced mitochondrial fusion when compared to the healthy pulpal tissue; 4) a higher rate of apoptosis was observed in inflamed pulpal tissues in comparison to healthy pulpal tissue. Therefore, we rejected our null hypothesis.

It has been widely reported that activation of the NF-κB signalling pathway triggers inflammation in pulpal tissues (24, 25). Previous studies found that TNF-α and NF-κB were upregulated in the inflamed human dental pulp and LPS-treated human dental pulp stem cells (2, 25, 26). Consistent with these findings, our results demonstrated that irreversible pulpitis had inflamed pulpal tissues, as indicated by greater levels of NF-κB and TNF-α, compared to the healthy pulp. TNF-α is one of the potent inflammatory cytokines that induce oxidative stress (27, 28). In line with the outcomes of our previous research, we found that oxidative stress occurred concurrently with inflammation in pulpitis.

Interestingly, we detected a higher expression of SOD2, the mitochondrial antioxidant, in irreversible pulpitis than in healthy pulpal tissues. These results were consistent with Bödör et al. (29), which reported a marked elevation of SOD2 levels in human irreversible pulpitis tissue. We have speculated that the increased SOD2 might be the compensatory response to oxidative stress in cases of irreversible pulpitis.

There is mounting evidence to show that inflammation and oxidative stress could induce cellular apoptosis (3, 15), findings consistent with our study where inflammation and oxidative stress occurred alongside apoptosis in irreversible

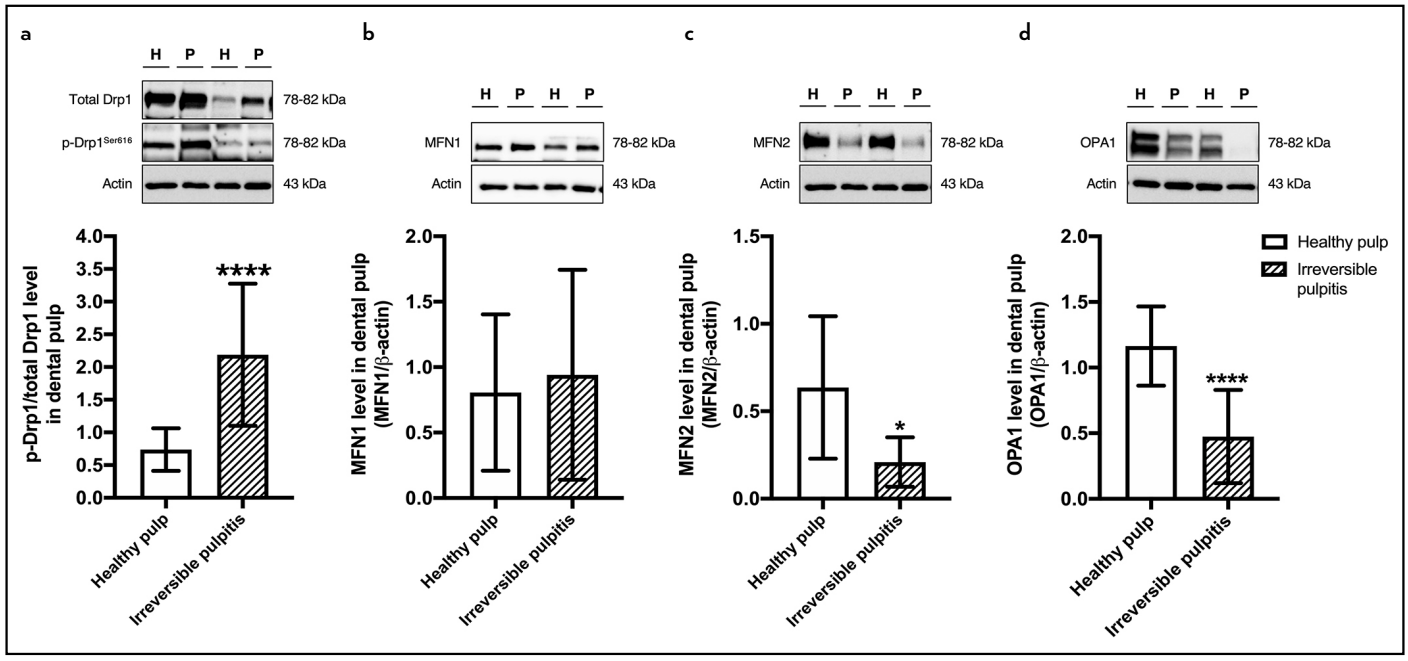

Figure 2. The illustration of markers of mitochondrial dynamics in irreversible pulpitis. The ratio of the expression of p-Drp1 to total-Drp1 (a, e), MFN1 (b, f), MFN2 (c, g), and OPA1 (d, h) in healthy human pulp and irreversible pulpitis samples

Data are shown as mean±SD (n=15). *p<0.05 and ****p<0.0001 denotes a statistically significant difference from the healthy pulp group. H: Healthy pulp, P: Irreversible pulpitis, p-Drp1: Phosphorylated-dynamin-related protein 1, MFN1: Mitofusin 1, MFN2: Mitofusin 2, OPA1: Optic atrophy type 1

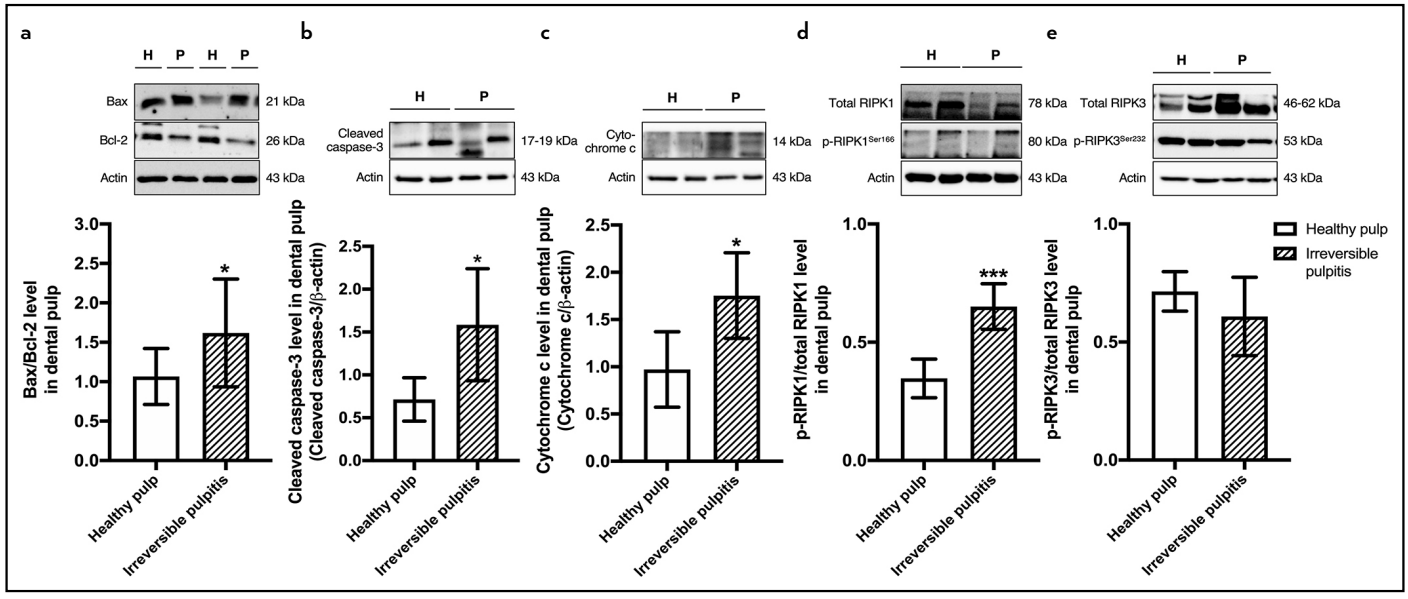

Figure 3. The evaluation of apoptosis and necroptosis in irreversible pulpitis. The ratio of the expression of Bax to Bcl-2 (a, f), cleaved caspase-3 (b, g), cytochrome c (c, h), p-RIPK1 divided by total RIPK1 (d, i), and p-RIPK3 divided by total RIPK3 (e, j) in healthy human pulp and irreversible pulpitis samples

Data are shown as mean±SD (n=15). *p<0.05 and ***p<0.001 denotes a statistically significant difference from the healthy pulp group. H: Healthy pulp, P: Irreversible pulpitis, Bax: Bcl-2-associated X protein, Bcl-2: B-cell lymphoma 2 protein, p-RIPK1: Phosphorylated-receptor-interacting serine or threonine-protein kinase 1, p-RIPK3: Phosphorylated-receptor-interacting serine or threonine-protein kinase 3

pulpitis tissue. In addition, TNF-α could trigger apoptosis and necroptosis through death receptors (25). This study showed that the apoptotic process was observed in inflamed pulpal tissues, as indicated by the increased ratio of Bax to Bcl-2 and increased cleaved caspase-3 and cytochrome c in inflamed pulpal tissues. We also found an increase in the ratio of the expression of the p-RIPK1 to total RIPK1 but not the ratio of p-RIPK3 to total RIPK3 of inflamed pulpal tissues. A previous study demonstrated that increased p-RIPK1 divided by total RIPK1 led to caspase-8 activation followed by apoptosis (30), which was in agreement with the outcome of our study. Therefore, our findings suggested that only apoptosis, but not necroptosis, was observed in inflamed pulpal tissues, a finding in agreement with a study by Lim et al. (31). The au-

thors demonstrated that differences in the levels of necroptotic marker RIP3 in irreversible pulpitis did not reach statistical significance when compared to the normal pulp (31).

In addition, the evidence available suggested that inflammation and oxidative stress could stimulate mitochondrial-dependent apoptosis (11). We found higher mitochondrial-dependent apoptotic markers in inflamed dental pulp tissues compared to healthy pulp tissues. Moreover, enhanced mitochondrial fission and poor mitochondrial fusion were observed in irreversible pulpitis tissues, compared to healthy pulp tissue, with findings similar to a previous study (2). It is possible that pulpal inflammation and oxidative stress in irreversible pulpitis led to a disturbance in the balance of mitochondrial dynamics and caused cellular apoptosis, possibly through the mitochondrial-dependent pathway. Therefore, targeting mitochondrial dynamics by maintaining the balance between mitochondrial fission and mitochondrial fusion may help improve cell survival and promote pulp regeneration in patients with irreversible pulpitis.

There were some limitations in this study. Specifically, the number of teeth included in this study was small, and there were statistically significant age differences between the healthy and the irreversible pulpitis groups. Consequently, these factors might weaken the findings of this study. Furthermore, since pulpal tissues from young permanent teeth were included in this study, this could also alter the results. However, future studies with larger sample sizes and a more standardised age profile are warranted to ensure the findings represent the main population.

CONCLUSION

We observed upregulation of the NF-κB signalling pathway in tissues from patients with irreversible pulpitis associated with an inflammatory cytokine release and oxidative stress. We assumed that inflammation and oxidative stress in cases of irreversible pulpitis might lead to changes in mitochondrial dynamics and cell death via apoptosis. The data support this assumption; however, it could make the findings less transferable to other situations.

Disclosures

Conflict of interest: The authors deny any conflict of interest.

Ethics Committee Approval: This study was approved by The Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand Human Experimentation Ethics Committee (Date: 22/10/2020, Number: 44/2020).

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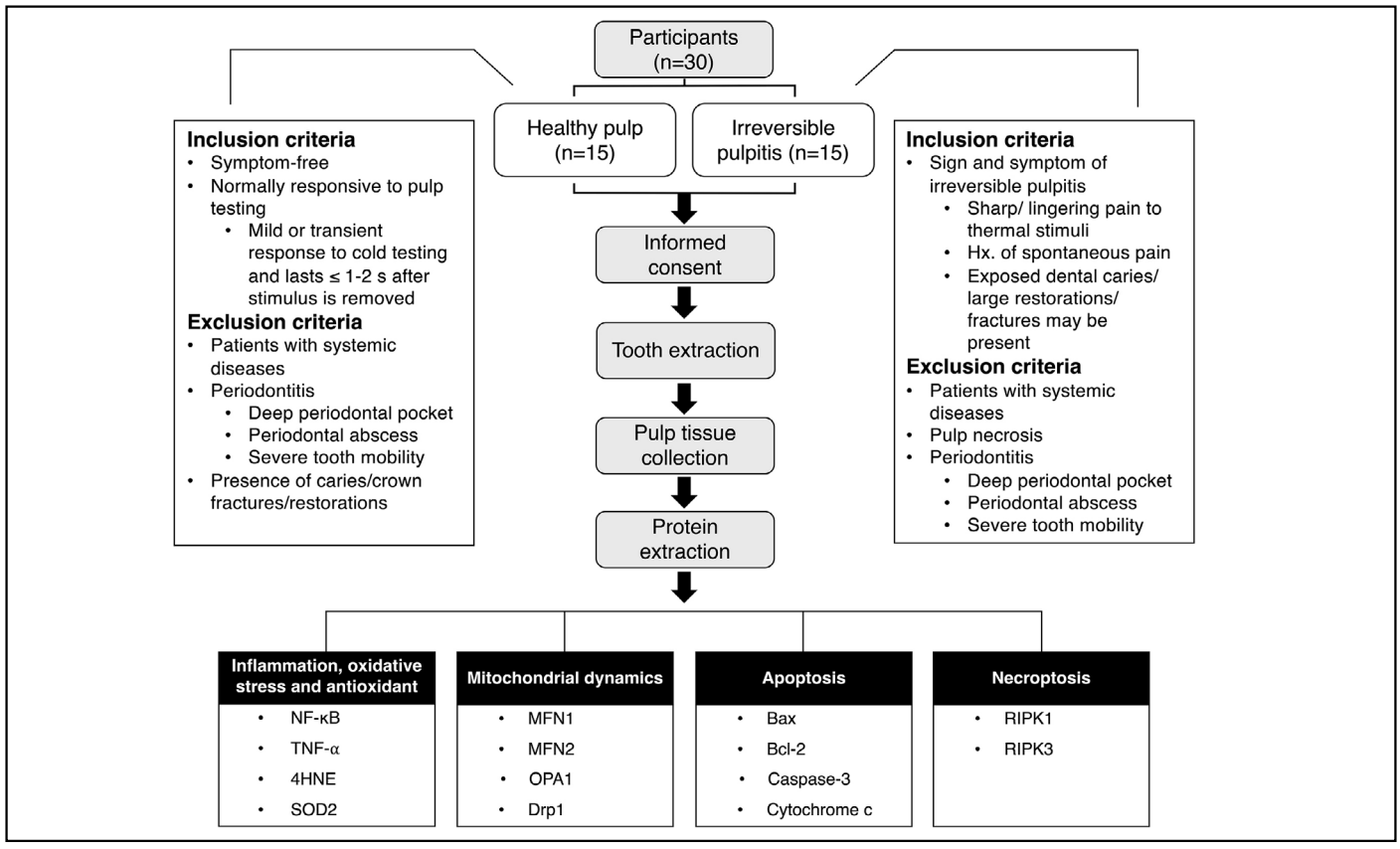
APPENDIX



Appendix 1. Diagram of the study protocol

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells, TNF-α: Tumour necrosis factor-alpha, 4HNE: 4 hydroxynonenal, SOD2: Superoxide dismutase 2, MFN1: Mitofusin 1, MFN2: Mitofusin 2, OPA1: Optic atrophy type 1, Drp1: Dynamin-related protein 1, Bax: Bcl-2-associated X protein, Bcl-2: B-cell lymphoma 2 protein, RIPK1: Receptor-interacting serine or threonine-protein kinase 1, RIPK3: Receptor-interacting serine or threonine-protein kinase 3