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Histology-based profile of inflammatory mediators in experimentally induced pulpitis in a rat model: screening for possible biomarkers

M. Li[1,2], J. Tian[1,2], Z. Xu[1,2], Q. Zeng[1,2], W. Chen[1,2], S. Lei[1,2] & X. Wei[1,2]

[1]Department of Operative Dentistry and Endodontics, Guanghua School of Stomatology, Hospital of Stomatology, Sun Yat-sen University, Guangzhou; and [2]Guangdong Provincial Key Laboratory of Stomatology, Guangzhou, China

Abstract

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Aim To profile molecular changes in lipopolysaccharide (LPS)-induced experimental pulpitis in a rat model and explore the feasibility of a molecular-based diagnostic strategy for pulpitis.

Methodology Seventy-three maxillary incisors of Sprague-Dawley rats were used to establish pulpitis models with LPS. Inflammatory grading was performed in four equal sections of the pulp divided from the injured site to the root apex. An antibody array was used to compare the expression of 67 molecules between control pulp and inflamed pulp 12 and 72 h after LPS application. The levels of differentially expressed molecules in the control and inflamed pulp (collected at 3, 6, 9, 12, 24 and 72 h after LPS treatment) were examined via ELISA, and correlations between inflammatory scores and molecule expression were assessed. The molecule distributions in the pulp were investigated by immunofluorescence staining. Data were analysed with paired *t*-test, one-way ANOVA, Kruskal–Wallis tests, and Spearman's and Pearson's correlations with significance set at $P < 0.05$.

Results Polymorphonuclear neutrophils were observed in the injured site 3 h after LPS stimulation. Inflammatory infiltration peaked at 12 h and was limited to the injured site with osteodentine deposition at 72 h. Thirteen molecules were significantly differentially expressed between the control and LPS-injured pulp. ELISA validated that tissue inhibitor of metalloproteinase-1 (TIMP-1) expression dramatically peaked at 12 h (compared with other time points, $P < 0.05$) and returned to baseline at 72 h. The TIMP-1 concentration was strongly correlated with inflammation severity in the apical three-quarters of the pulp, and the strongest correlation was found in the lower-middle quarter ($r = 0.786$, $P < 0.001$). Immunofluorescence staining revealed that in the apical three-quarters of the pulp, TIMP-1 expression was significantly higher in the 12 h group than in the control and 3, 6, 24 and 72 h groups ($P < 0.01$).

Conclusion This study provides a molecular profile of LPS-induced pulpitis in a rat model. TIMP-1 had a strong positive correlation with the severity of dental pulp inflammation, verifying the feasibility of applying biomarkers to identify specific pathological conditions in pulpitis.

Correspondence: Xi Wei, Department of Operative Dentistry and Endodontics, Guanghua School of Stomatology, Hospital of Stomatology, Sun Yat-sen University, 56 Ling Yuan Xi Road, Guangzhou 510055, China (Tel.: +86-20-8388-0049; fax: +86-20-8382-2807; e-mail: weixi@mail.sysu.edu.cn).

Mengjie Li and Jun Tian contributed equally to this study.

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Introduction

Accurately diagnosing the inflammatory state of the dental pulp is crucial for making appropriate treatment decisions, especially when the extension of vital pulp therapy has been proposed to maintain pulpal health (ESE 2019). When inflammation is limited or reversible, vital pulp therapy can be a viable alternative to root canal treatment for the inflamed pulp (Schmalz & Smith 2014, Wolters *et al.* 2017). For teeth with deeply inflamed pulps, root canal treatment remains a therapeutic choice to eliminate pulpal infection and save teeth (Galani *et al.* 2017). However, it is not always easy to determine the pulpal status based only on clinical symptoms and radiological observations since there are various degrees of inflammation, ranging from mild to more severe (Yoon *et al.* 2010). In addition, whilst the concepts of 'reversible pulpitis' and 'irreversible pulpitis' remain clinically useful diagnostic terms, it is accepted that these terms simply reflect the operational intention of clinicians to maintain or remove the vital pulp (Zanini *et al.* 2017). Therefore, there have been calls to update classifications of pulpitis by associating clinical entities with pathological entities (ESE 2019). Currently, the European Society of Endodontology has approved the definition 'partial irreversible pulpitis' to more accurately reflect the histologic picture of pulp inflammation confined to the coronal tissues and vital pulp preserved in the radicular tissues (ESE 2019). Hence, the identification of biomarkers for pulpal inflammation is urgently needed to help improve clinical decision-making and predict the treatment outcomes of vital pulp therapy (Duncan *et al.* 2019).

Immune responses are one of the main factors involved in inflamed pulp tissues (Khorasani *et al.* 2020). A vast number of inflammatory mediators, such as interleukins (ILs), chemokines and tumour necrosis factors (TNFs), are implicated in immune responses (Monastero & Pentyala 2017). Furthermore, the quality and quantity of these mediators are key in the development of inflammation, especially in terms of the type of immune response provoked (Hirsch *et al.* 2017). Accordingly, given the important roles of various molecules involved in immune responses, it could be enlightening to study the profile of inflammatory mediators associated with pathological changes in pulpitis. To date, more than 89 inflammatory mediators have been assessed within human dental pulp tissues with various degrees of inflammation (Rechenberg *et al.* 2016). A systematic review revealed that IL-8, TNF-α, and matrix metalloproteinase-9 expression increased at both the gene and protein levels in teeth clinically diagnosed with irreversible symptomatic pulpitis (Zanini *et al.* 2017). In addition, the levels of some repair-enhancing molecules were upregulated in pulp tissue with high healing potential. For example, it has been reported that in samples of reversible pulpitis, osteocalcin and several angiogenic factors, such as vascular endothelial growth factor, fibroblast growth factor and platelet-derived growth factor, had marked increases in expression compared with normal pulp or irreversible pulpitis samples (Abd-Elmeguid *et al.* 2013, Emilia & Neelakantan 2015). The findings of previous studies have suggested potential correlations between specific levels of inflammatory mediators and histopathological conditions of injured pulp.

These measurable inflammatory mediators might serve as possible biomarkers to differentiate certain states of pulpal inflammation. Although previous studies have reported the existence of potential biomarkers for clinically diagnosed pulpitis, direct correlations between biomarkers and histologic diagnoses have not been established, as the histologic and clinical classifications of pulpitis still need to be matched and refined. No specific study has been performed to investigate biomarkers for pulpal inflammation based on histologic characteristics in either clinical samples or animal models. Currently, experimentally induced pulpitis in a rat model is widely used, as it is relatively available and inexpensive. In this study, the expression of 67 inflammatory mediators was analysed in experimentally induced pulpitis in a rat model and the findings were combined with histologic observations, aiming to profile the molecular changes in pulpal inflammation and evaluate the correlations between the expression of specific molecules and the severity of pulpal inflammation, thus exploring the feasibility of a molecular-based diagnostic strategy for pulpitis.

Materials and methods

Experimental pulpitis model

All procedures involving experimental animals followed the Animal Ethics Procedures and Guidelines of the People's Republic of China, and were approved by the Institutional Animal Care and Use Committee of Sun Yat-Sen University (IACUC No. DD-18-0110). All experiments were carried out with 8-week-old male Sprague-Dawley rats weighing 250–300 g (73 rats in total). The rats were housed under standard controlled conditions and were fed a pelleted rat diet *ad libitum*, and a qualified veterinarian conducted all the nutritional recommendations and was in charge of the care of the rats. The number of rats in each group was based on data from a previous study (Renard *et al.* 2016). Experimental pulpitis was induced by treating pulp tissues with bacterial lipopolysaccharide (LPS; derived from *Escherichia coli* O111:B4; Sigma Chemical Co., St. Louis, MO, USA), according to a modified method (Kawashima *et al.* 2005). Animals were anaesthetized with sodium pentobarbital (40 mg kg^{-1} body weight) via intraperitoneal injection, and local anaesthesia was applied to the surgical site using 2% lidocaine with epinephrine 1 : 100 000 (4.4 mg kg^{-1} $dose^{-1}$). The crown of the maxillary incisor was removed horizontally to expose the pulp chamber. Pupal exposure was confirmed by using a sterile size 10 K-file and sequentially enlarging the root canal to a size 40-K file with a 4 mm working length. After haemostasis was achieved, a sterile paper point, cut to 4 mm in length and soaked in five microlitres of phosphate-buffered saline (PBS) or LPS (10 mg mL^{-1}), was inserted into the prepared space. The cavities were filled with glass–ionomer cement (DMG, Hamburg, Germany). During the postoperative period, the health status of the rats (behaviour, body weight, changes in the skin and hair, food and water consummation, urinating and defecation) was checked daily. Teeth were collected at 3, 6, 9, 12, 24 and 72 h after PBS/LPS treatment. Normal pulps were collected in a similar manner from intact incisors as the control group.

Histologic analysis

Teeth were fixed in 4% paraformaldehyde at 4 °C for 24 h, decalcified with 10% ethylenediaminetetraacetic acid for 8 weeks, embedded in paraffin and sectioned at 4 μm along the labial-palatal plane for haematoxylin and eosin (HE) staining ($n = 4$ per group). The largest section of each sample containing the maximum area of the injury site was selected for qualitative and quantitative histologic analysis.

When assessing the extent and intensity of inflammatory infiltration in injured pulp, the entire pulp, from the injured site to the apex of the pulp, was divided into by length, which were categorized as the coronal, upper-middle, lower-middle and apical quarters of the pulp. In each section of the pulp, 4 high-magnification fields of vision were randomly selected for inflammation grading. The density of polymorphonuclear neutrophils (PMNs) in each part of the pulp was graded based on modified scoring criteria as follows (Ying *et al.* 2017): absent (score 0), 1–20 PMNs (score 1), 21–40 PMNs (score 2), 41–80 PMNs (score 3) and >80 PMNs (score 4). Two experienced examiners blinded to the groupings evaluated the samples. Before grading, the interrater reliability test showed that the intraclass correlation coefficient [95% CI] was 0.872 [0.819, 0.910] ($P < 0.001$), demonstrating excellent consistency between the two examiners. For sections with disagreement, the examiners arrived at a consensus after deliberation. Other histologic features, such as hyperaemia, blood vessel dilation, tissue disorganization and hard tissue formation in the pulp, were also recorded.

Preparation of the supernatants of pulp tissues

The pulp tissues collected from the control group and LPS groups were immediately weighed. Tissues were mixed with cell lysis buffer and protease inhibitor (RayBiotech Inc., Norcross, GA, USA), and fragmented for 60 s by a tissue disintegrator. After incubated for 45 min on ice, the mixtures were centrifuged at 30 000 ***g*** for 15 min at 4 , then the supernatants were collected and stored at −80 for further tests. The total proteins in the supernatants were quantified using the bicinchoninic acid assay protein assay kit (Biocolor Bioscience & Technology, Shanghai, China) following the vendor's instruction.

Antibody arrays

HE staining revealed that the pulp tissue 12 h after LPS treatment had the most severe signs of inflammation, whilst the pulp at 72 h after LPS treatment had limited inflammation with emerging tissue repair events. Hence, the supernatants of LPS-treated pulp

samples at 12 and 72 h and the control pulp were collected for an assay ($n = 3$ per group). The relative concentrations of a total of 67 proteins were measured with the Rat Cytokine Antibody Array Kit (GSR-CAA-67-1; RayBiotech Inc.) to identify differential molecules. The relative expression profiles of proteins are presented as a heatmap that was generated using R software (http://www.r-project.org/). In the screening stage, the molecules were considered differential proteins if the signal intensity exceeded 150 and the fold change was not less than 1.2-fold ($P < 0.05$) in the pairwise comparison. Differential proteins with the highest fold changes were chosen for verification by enzyme-linked immunosorbent assay (ELISA).

ELISA

Tissue inhibitor of metalloproteinase-1 (TIMP-1) and β-nerve growth factor (β-NGF), two of the 13 identified differential proteins, were chosen for validation and further analysis. The levels of the selected proteins in control and inflamed pulp (collected at 3, 6, 9, 12, 24 and 72 h after LPS treatment, $n = 4$ per group) were serially examined using commercially available ELISA kits (RayBiotech Inc.). The final concentrations of proteins were reported as pictograms per millilitre (pg mL^{-1}) based on a total protein concentration of 500 mg mL^{-1}.

Immunofluorescence staining

Pulp tissues were fixed with 4% paraformaldehyde overnight at 4 °C, embedded in optimal cutting temperature compound (OCT; Sakura Finetek, Torrance, CA, USA) and then cut into 8-μm-thick sections along the labial-palatal plane ($n = 3$ per group). The frozen sections were air-dried for 30 min, washed with PBS, permeabilized with 0.1% Triton X-100, and then blocked with 5% BSA for 60 min. Then, the sections were incubated with an anti-TIMP-1 rabbit polyclonal antibody at a dilution of 1 : 400 (Bioss, Wuhan, China) overnight at 4 °C. After washing with PBS, the sections were incubated with an Alexa Fluor 568-conjugated secondary antibody (1 : 1000; Invitrogen, Waltham, MA, USA) for 60 min at room temperature. The slides were mounted with FluoroShield mounting medium containing 4′,6-diamidino-2-phenylindole (Abcam, Cambridge, UK). Images were obtained using a fluorescence microscope (Carl Zeiss Meditec AG, Jena, Germany), and the fluorescence was quantified using ImageJ software (Image J 1.51v, National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Data were analysed using Prism 7 (GraphPad Software Inc., San Diego, CA, USA). Quantitative data are presented as the mean ± SD. One-way ANOVA or Kruskal–Wallis tests with the Bonferroni correction were used to compare data from more than two groups, as appropriate. Spearman's correlation or Pearson's correlation, as indicated in the figure legends, was used to assess correlations between the expression of inflammatory mediators and the degree of pulpal inflammation. A paired *t*-test was used for statistical analysis of paired groups. For all analyses, statistical significance was set at $P < 0.05$.

Results

This animal study was reported according to the PRIASE 2021 Guidelines (Fig. 1; Nagendrababu *et al.* 2021). The general health condition of the rats remained constant throughout the experimental period. At the end of the experimental period, no significant difference was observed in mean body weight amongst the groups (data not shown).

Histologic findings for experimentally induced pulpitis in rats

HE staining revealed that the control pulps had no pathological changes in the dentine/predentine/odontoblast complex or inflammatory cell responses (Fig. 2a).

In teeth treated with PBS, no inflammatory cells were found in the pulp at 3 h (Fig. S1a). At 6 h, scattered PMNs and slightly dilated blood vessels were observed in close proximity to the injured site (Fig. S1b). Then, increasing numbers of inflammatory cells infiltrated beneath the injured site from 9 h until 24 h post-injury (Fig. S1c–e). In addition, a disturbed odontoblast layer and red blood cell extravasation were observed below the injured site. The inflammatory cell responses peaked at 24 h after PBS treatment, exhibiting a moderate density of PMNs dispersed throughout the coronal quarter of the pulp. Subsequently, inflammation tended to subside. At 72 h, inflammatory cells were limited to the pulp horn with surrounding thin fibres and scattered hard tissue deposition, whilst the rest of the pulp had recovered from inflammation (Fig. S1f).

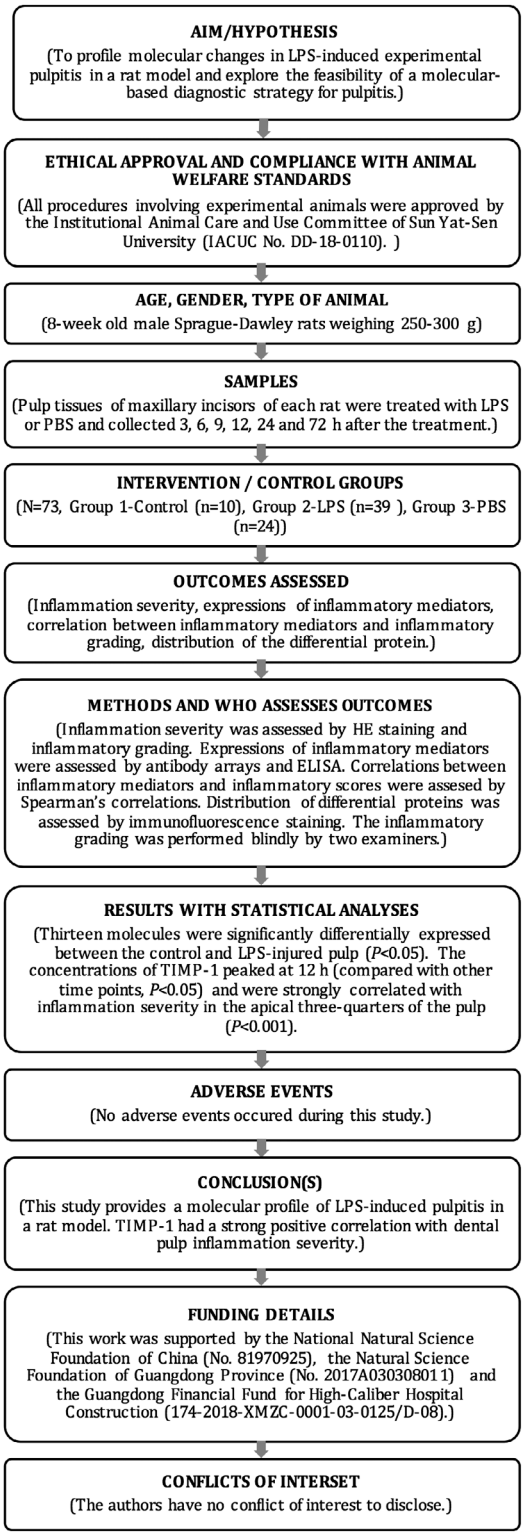

Figure 1 PRIASE 2021 flowchart illustrating the steps involved in conducting the present study.

In comparison, teeth treated with LPS had more severe and rapid signs of pulpal inflammation (Fig. 2). Rapid recruitment of PMNs was observed through dilated blood vessels as early as 3 h (Fig. 2b). From 6 to 12 h, the odontoblast layers in the vicinity of the injured site were reduced in number and flattened, and hyperaemia was found in the upper half of the pulp. In addition, an increasing number of inflammatory cells accumulated in the injured site and infiltrated towards the apex of the pulp (Fig. 2c–e). The inflammatory infiltration at 12 h post-injury appeared to be the most extensive, presenting as dispersed inflammatory cells throughout the majority of the pulp (Fig. 2e-2). After 12 h, whilst masses of inflammatory cells consistently infiltrated the injured site (Fig. 2f–g), the density of inflammatory cells in the apical three-quarters of the pulp began to decrease. At 72 h after LPS treatment, the histologic condition of the apical three-quarters of the pulp was similar to that of the normal pulp, which was characterized by inflammatory cell absence and well-organized pulp tissues (Fig. 2g-2). Notably, a slight layer of newly generated hard tissue deposition and dense fibre bundles underneath the intensive PMN halo were observed at 72 h (Fig. 2g-1).

The distributions of inflammatory scores are shown in Table 1. Inflammatory cells intensively accumulated in the coronal quarter of the pulp from 12 to 72 h after LPS stimulation, with the LPS-injured pulp exhibiting higher scores than the control pulp ($P < 0.05$). In the upper-middle quarter of the pulp, the inflammatory scores for the LPS-treated pulp at 9, 12, and 24 h were significantly higher than those for the control pulp ($P < 0.05$). In the lower-middle quarter of the pulp, the inflammatory scores peaked at 12 h and were significantly higher than those of the control pulp and the 3 h LPS-treated pulp ($P < 0.05$). Moreover, the inflammatory score for the apical quarter of the pulp at 12 h was significantly higher than that of the control pulp as well as those of the LPS-treated pulp at 3, 6 and 72 h ($P < 0.05$). Quantitative analysis verified that inflammatory infiltration peaked at 12 h after LPS stimulation. In addition, the inflammation progression pattern in the coronal quarter was different from that in the apical three-quarters of the pulp.

Expression of inflammatory mediators in inflamed pulp tissues in rats

The expression profiles of 67 inflammatory mediators in the control pulp and inflamed pulp 12 and 72 h

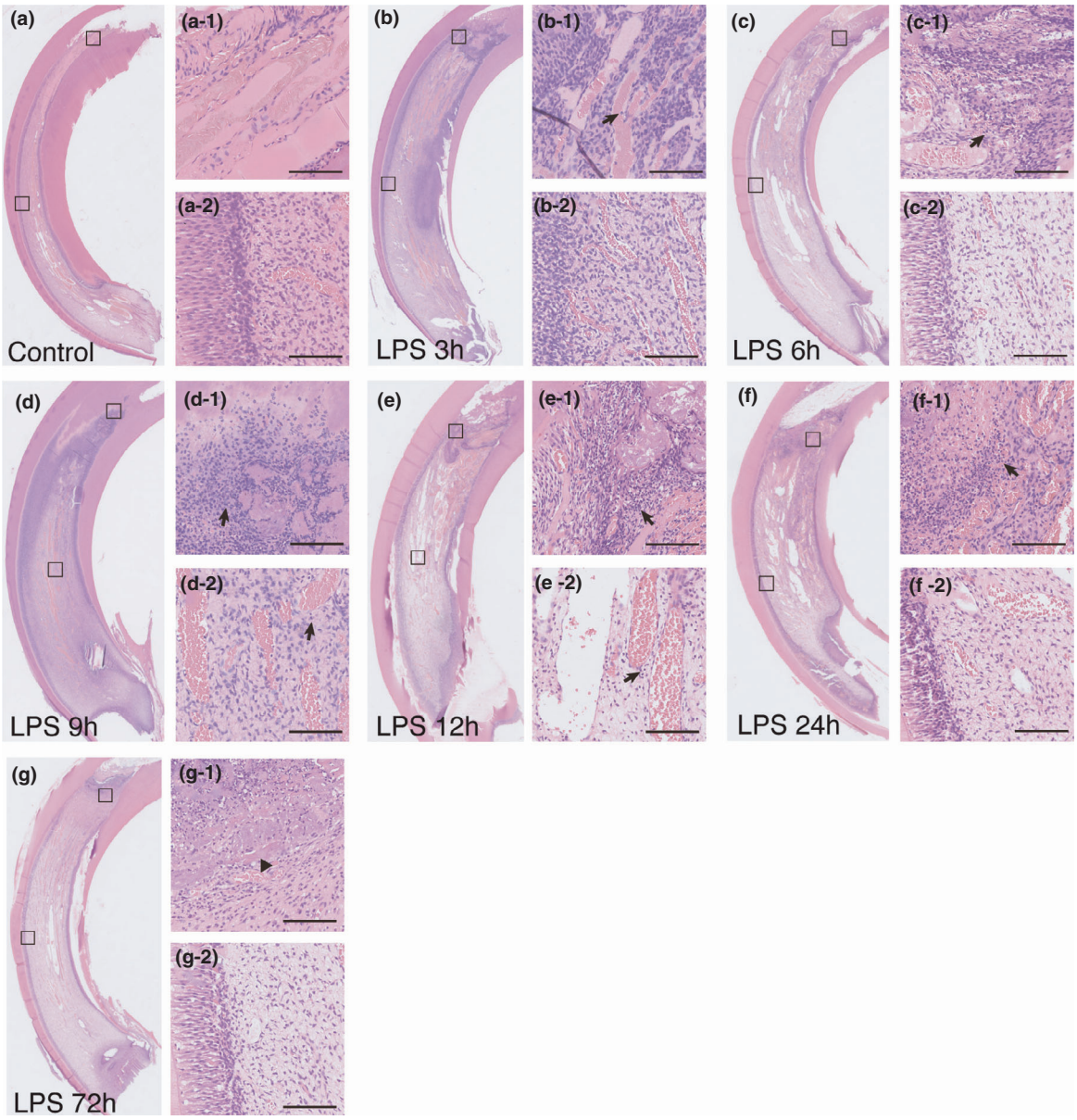

Figure 2 Inflammatory infiltration in control pulp and LPS-injured pulp. (a–g) Representative HE staining images of inflammatory infiltration in pulp tissues of maxillary incisors are shown (scale bar: 100 μm). (a) In the control pulp, no inflammatory cell infiltration was observed. (b) At 3 h, a few polymorphonuclear neutrophils (PMNs) were attracted to the injured site through dilated blood vessels. (c) At 6 h, PMNs were scattered under the injured site. The structure of the pulp tissues in the lower half of the pulp was not disturbed. (d) At 9 h, PMNs were moderately concentrated beneath the injured site and infiltrated towards the apex of the pulp via dilated blood vessels. (e) At 12 h, PMNs were intensively concentrated in the coronal quarter of the pulp and moderately infiltrated into the rest of the pulp. Inflammatory cells were mainly scattered around the vessels in the apical three-quarters of the pulp. (f) At 24 h, PMNs were aggregated beneath the injured site but were rarely found in the apical three-quarters of the pulp. (g) At 72 h, PMNs in the coronal quarter of the pulp were surrounded by hard tissue depositions and fibre bundles. The arrows show the infiltration of PMNs. The arrow head shows osteodentine deposition around the injured site of the pulp.

after LPS treatment are displayed in Fig. 3a, and 13 molecules were found to be significantly different between the control pulp and LPS-injured pulp (Fig. 3b). Amongst the 13 proteins, seven including TIMP-1, β-NGF, IL-10, galectin-3, intercellular adhesion molecule 1 (ICAM-1), fractalkine (CX3CL1) and notch-2 were upregulated in the inflamed pulp. In addition, six including TNF-like weak inducer of apoptosis receptor (TWEAK-R), T cell immunoglobulin mucin domain-1 (TIM-1), prolactin, neighbour of punc E11 (Nope), P-cadherin and tissue inhibitor of metalloproteinase-2 (TIMP-2) were downregulated in the inflamed pulp compared with the control pulp. Notably, amongst those of the abovementioned proteins, the signal intensities of TIMP-1 and β-NGF had the greatest differences amongst the three groups (Fig. 3c). The relative expression profile revealed that the level of TIMP-1 in the inflamed pulp 12 h after LPS treatment was three-fold higher than that in the control pulp and 1.6-fold higher than that in the inflamed pulp 72 h after LPS treatment ($P < 0.05$). In addition, the level of β-NGF in the inflamed pulp 12 h after LPS treatment was 2.9 times higher than that in the control pulp and 2.1 times higher than that in the inflamed pulp 72 h after LPS treatment ($P < 0.05$).

Table 1 Percentages of inflammatory scores for LPS-induced pulpitis (%)

	Inflammatory grading for the coronal 1/4 of the pulp					Inflammatory grading for the upper-middle 1/4 of the pulp				
Group	0	1	2	3	4	0	1	2	3	4
Control	100	0	0	0	0	100	0	0	0	0
3 h	0	100	0	0	0	50	50	0	0	0
6 h	0	0	100	0	0	0	50	50	0	0
9 h	0	0	0	100	0	0	0	75	25	0
12 h	0	0	0	25	75	0	0	50	50	0
24 h	0	0	0	100	0	0	0	75	25	0
72 h	0	0	0	25	75	0	75	25	0	0
	Inflammatory grading for the lower-middle 1/4 of the pulp					Inflammatory grading for the apical 1/4 of the pulp				
Group	0	1	2	3	4	0	1	2	3	4
Control	100	0	0	0	0	100	0	0	0	0
3 h	0	100	0	0	0	50	50	0	0	0
6 h	0	0	100	0	0	0	50	50	0	0
9 h	0	0	0	100	0	0	0	75	25	0
12 h	0	0	0	25	75	0	0	50	50	0
24 h	0	0	0	100	0	0	0	75	25	0
72 h	0	0	0	25	75	0	75	25	0	0

The levels of TIMP-1 and β-NGF were determined by ELISA at different time points during LPS-induced pulpitis (Fig. 3d–e). The level of TIMP-1 increased dramatically to the peak level at 12 h (compared with the other time points, $P < 0.05$). In addition, the level of β-NGF peaked at 24 h in the inflamed pulp, and the 24-h level was significantly higher than that in the control pulp and the 72 h LPS-treated pulp group ($P < 0.05$ in the control group, $P < 0.01$ in the 72 h LPS-treated group). At 72 h, the concentrations of the two proteins in the inflamed pulp had declined and were not significantly different from those in the control pulp ($P > 0.05$).

Correlations between biomarker levels and pulp inflammation

The correlation coefficients between the levels of TIMP-1 or β-NGF and inflammatory scores are shown in Fig. 4. The concentration of TIMP-1 was strongly correlated with inflammation severity in the apical three-quarters of the pulp ($P < 0.001$, Fig. 4b–d), and the strongest correlation was found in the lower-middle quarter of the pulp ($r = 0.786$, $P < 0.001$, Fig. 4c). In addition, a weak positive correlation between TIMP-1 and the inflammatory scores in the coronal quarter of the pulp was found ($r = 0.394$, $P < 0.05$, Fig. 4a). The correlations between the levels of β-NGF and inflammatory scores were relatively weak in the upper-middle quarter and lower-middle quarter of the pulp ($r = 0.475$ and 0.472, respectively, $P < 0.05$, Fig. 4f-g), whereas the correlations in the coronal and apical quarters were not significantly different (Fig. 4e,h). In addition, Pearson's correlation analysis revealed a moderate positive correlation between TIMP-1 and β-NGF ($r = 0.508$, $P < 0.01$, Fig. S2). Considering the significant change in the concentration of TIMP-1 as well as its strong correlation with inflammation severity, TIMP-1 was selected for further study.

Distribution of TIMP-1 in inflamed pulp tissues

To further demonstrate the correlation between TIMP-1 and the severity of pulpal inflammation, the distribution of TIMP-1 in the entire pulp tissue was investigated by immunofluorescence staining. Immunofluorescence staining revealed positive expression of TIMP-1 in pulp tissues stimulated with LPS (Fig. 5). At 3, 6, 9, 12 and 24 h after LPS treatment, the level of TIMP-1 in the apical quarter of the pulp was higher than that in the coronal quarter, whilst

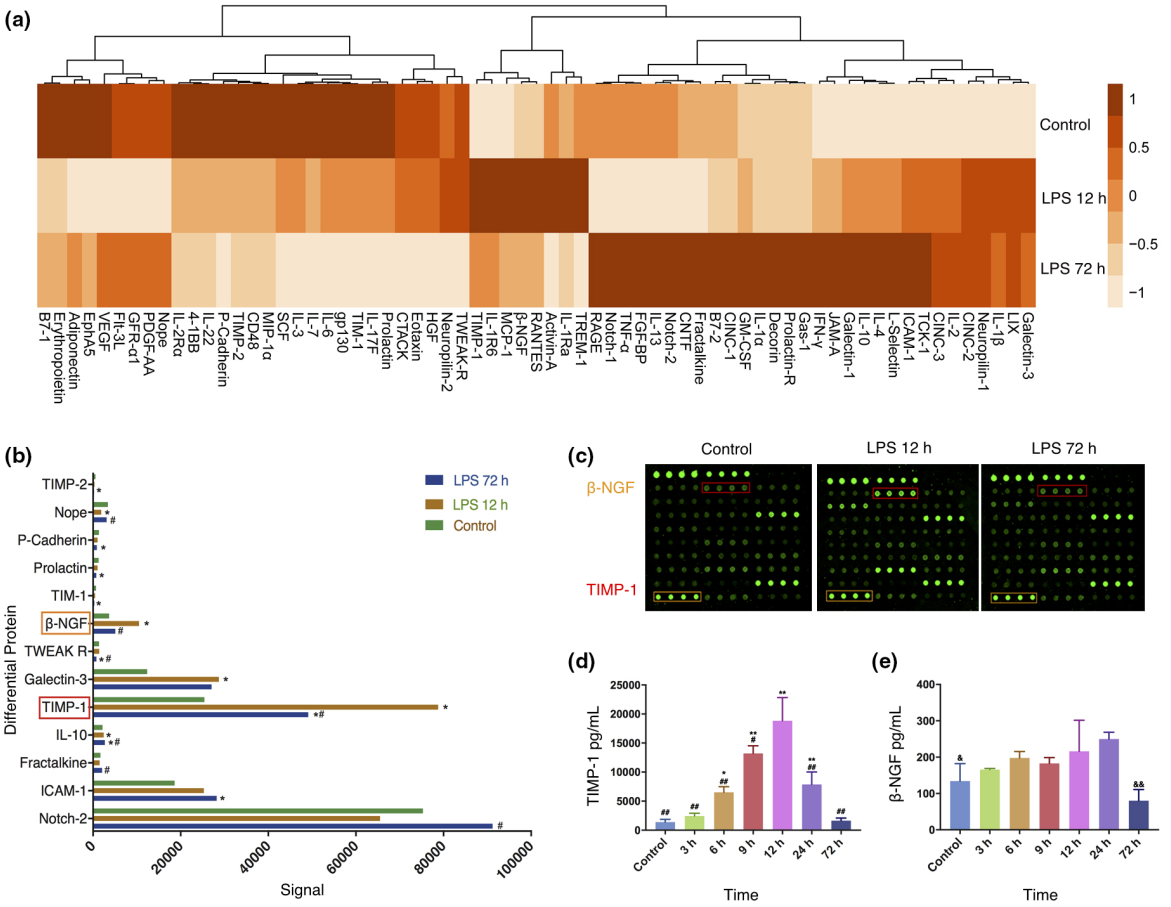

Figure 3 Profile of inflammatory mediators in LPS-induced experimental pulpitis. (a) The cluster heatmap shows the relative expression profile of inflammatory mediators in the control pulp and inflamed pulp assessed by antibody array analysis. (b) There were 13 molecules identified to be significantly different in multiple comparisons amongst the control pulp and inflamed pulp treated with LPS for 12 h or 72 h. (c) Fluorescence signals showing the differences in TIMP-1 or β-NGF between the control and LPS-treated pulp. (d) The levels of TIMP-1 in control and LPS-treated inflamed pulp were determined using ELISA kits. (e) The levels of β-NGF in control and LPS-treated inflamed pulp were determined using ELISA kits. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with the control; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared with the 12 h LPS-treated group; $^{\&}P < 0.05$ and $^{\&\&}P < 0.01$, compared with the 24 h LPS-treated group.

statistical significance was found only at 6 h post-injury ($P < 0.05$, Fig. 5f). Moreover, the expression of TIMP-1 in the apical three-quarters of the pulp in the 12 h LPS-treated group was significantly higher than that in the control group and the 3, 6, 24 and 72 h LPS-treated groups ($P < 0.01$, Fig. 5g). However, although the expression of TIMP-1 in the coronal quarter of the pulp peaked at 12 h, no significant difference was found amongst the groups ($P > 0.05$).

Discussion

In this study, a model of LPS-induced incisor rat pulpitis was established as with previous studies to explore the histologic and molecular changes in acutely inflamed pulp tissues (Kawanishi *et al.* 2004, Kawashima *et al.* 2005, Ohkura *et al.* 2014, Renard *et al.* 2016). Previously, investigators reported the proportions of several immune cells (granulocytes, macrophages, dendritic cells, etc.) in rat incisors inflamed by LPS, noting that the analysis of the immune response based on this model is a first step towards the identification of molecular or cellular targets for new therapeutic agents in mild reversible pulpitis in immature teeth (Renard *et al.* 2016). Furthermore, this model was chosen because rat incisors can provide sufficient dental pulp tissues for antibody arrays, which would be less possible with

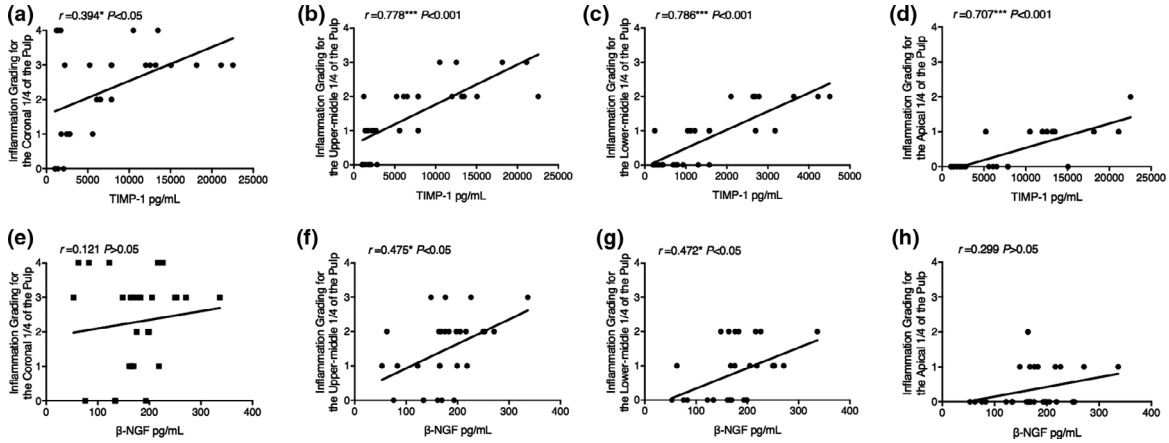

Figure 4 Correlations of the levels of TIMP-1 or β-NGF with the severity of pulp inflammation. (a–c) Spearman's correlation analysis of the levels of TIMP-1 and inflammatory scores for the coronal quarter, upper-middle quarter, lower-middle quarter or apical quarter of the pulp. (d–f) Spearman's correlation analysis of β-NGF levels and inflammatory scores for the coronal quarter, upper-middle quarter, lower-middle quarter or apical quarter of the pulp. *r*, correlation coefficient. $*P < 0.05$, $***P < 0.001$.

molars. LPS is a component of the cell wall of gram-negative bacteria, which commonly participates in endodontic infections and is widely used to induce inflammatory reactions in dental pulp tissues *in vivo* (Ning *et al.* 2020). Pulp exposure with subsequent bacterial infection from the oral environment has also been a common method to induce pulpal inflammation, and this method is more rapid and convenient than LPS stimulation. In addition, several studies have applied specific bacteria to induce pulpal inflammation; however, they reported that in rat incisors, the proportions of T lymphocyte subsets can change depending on the species of bacteria (Kim & Lim 2002), suggesting that differences in the bacteria used might lead to the discrepancies in molecular profiles amongst subjects. Moreover, for the pulp exposure model, there is a risk of exposure to unknown sources of inflammation by pulp exposure. Taking all these factors into consideration, the LPS-induced rat incisor pulpitis model was applied in the present study.

The histologic findings revealed that pulpal inflammation peaked at 12 h after LPS treatment, presenting as a massive infiltration of PMNs. Similar to the events during wound infections occurring at other sites, PMNs are recruited abundantly and provide the first line of defence in pulp tissue (Cooper *et al.* 2017). Neutrophil infiltration has been reported to be the key feature in defining the severity of pulp lesions in acute pulpitis in humans (Giuroiu *et al.* 2015). On the one hand, neutrophils help clear infections by engulfing and destroying invading microorganisms. However, on the other hand, activated neutrophils release large amounts of reactive oxygen species and matrix metalloproteinases (MMPs), thus leading to irreversible pulp destruction (Farges *et al.* 2015, Zanini *et al.* 2017). At the microscopic level, the most severe sign of inflammation was observed at 12 h when extensive PMN infiltration, remarkable blood vessel dilation and a disordered odontoblast layer were present. At the molecular level, the pro-inflammatory mediator galectin-3 showed significantly higher expression in the pulp at 12 h after LPS treatment than in the control pulp. As a member of the β-galactoside-binding lectin family, galectin-3 can facilitate neutrophil activation, adhesion and migration (Sciacchitano *et al.* 2018). Interestingly, the significant increases in TIMP-1 and IL-10 levels in the inflamed pulp at 12 h indicated the initiation of pulp tissue repair as pulpal inflammation progressed. In addition, the expression of β-NGF at 12 h was significantly higher than that in the control pulp. Previous studies have demonstrated that the expression of β-NGF is strongly upregulated in odontoblasts facing decay (Mitsiadis *et al.* 2017a,b). Moreover, β-NGF can induce odontoblast differentiation and is implicated in dentinogenesis, suggesting that β-NGF signalling may play a role in pulp tissue repair (Arany *et al.* 2009, Mitsiadis *et al.* 2017a,b). In general, the LPS-injured pulp at 12 h was deeply inflamed, but the intracellular molecular cascade of the reparative response was activated by the

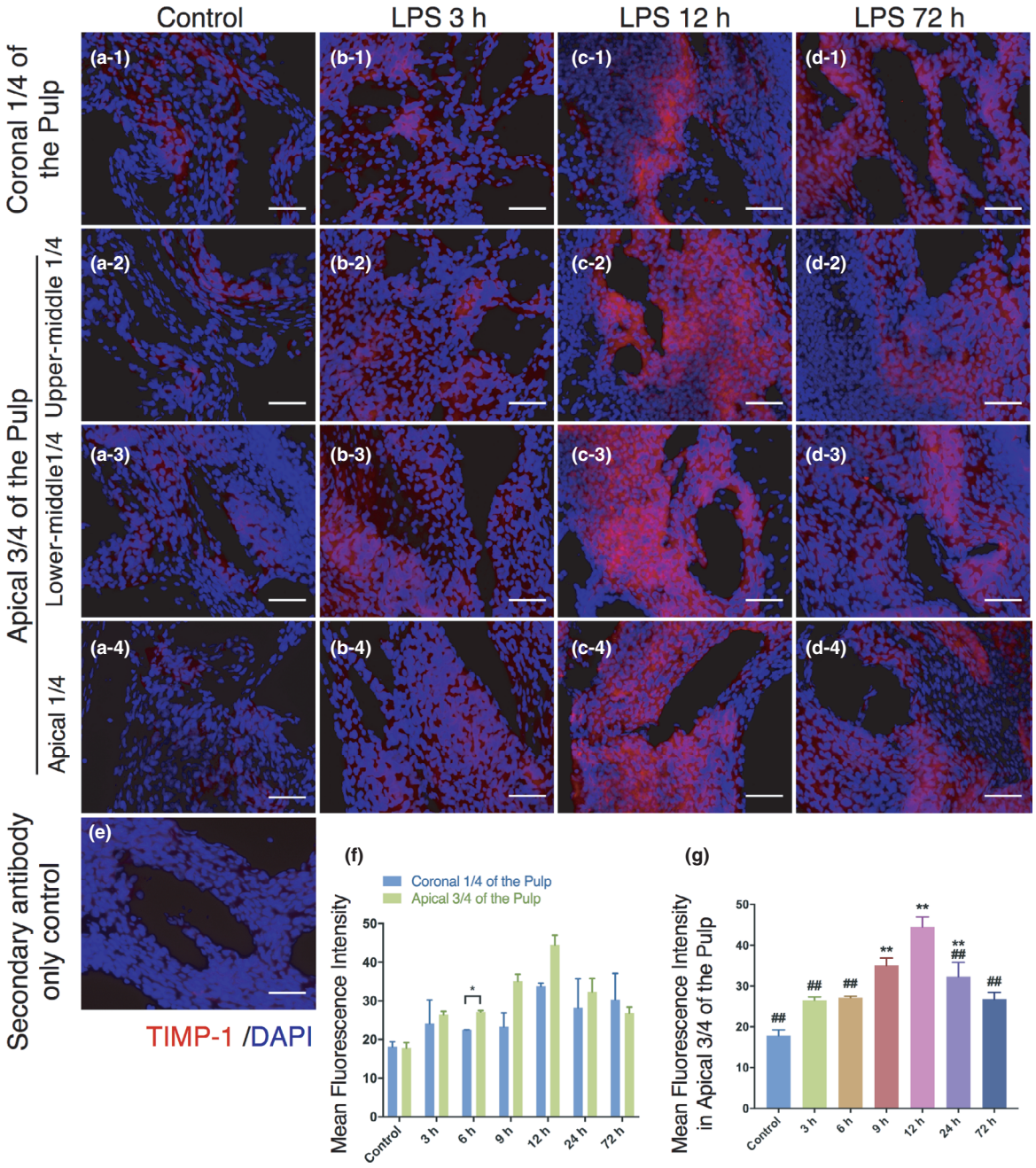

Figure 5 The expression and distribution of TIMP-1 in LPS-induced pulpitis. (a-d) Representative immunofluorescence images of TIMP-1 in the control pulp and LPS-treated inflamed pulp of maxillary incisors. (e) Immunofluorescence image of the secondary antibody only control (scale bar: 50 μm). (f) Comparison of TIMP-1 fluorescence intensity between the coronal quarter and the apical three-quarters of the pulp. $^{*}P < 0.05$. (g). Quantification of TIMP-1 fluorescence intensity in the apical three-quarters of the pulp in control teeth and LPS-treated teeth. $^{**}P < 0.01$, compared with the control; $^{\#\#}P < 0.01$, compared with the 12 h LPS-treated group.

inflammatory signals, as the expression of factors involved in anti-inflammatory or tissue repair events was significantly upregulated.

HE staining revealed that at 72 h, inflammatory cells were confined to the injured site, whilst the apical three-quarters of the pulp closely resembled

normal pulp tissue, suggesting that in some cases of pulpitis, the pulp may be partially inflamed whilst the rest of the vital pulp is preserved. In addition, tissue repair events, such as fibrosis and hard tissue formation, occurred in the pulp. Denser fibre bundles and more osteodentine deposition around the injured site were observed in the LPS-injured pulp than in the PBS-injured pulp at 72 h, indicating that enhanced reparative activity occurred at the inflamed site. In addition, a previous study reported that greater osteodentine deposition occurred in LPS-treated rat incisors than in PBS-treated rat incisors (Renard *et al.* 2016). The reparative response can be activated by inflammation-related molecules, including LPS, cytokines and reactive oxygen species (Farges *et al.* 2015). LPS at low concentrations (0.1–1 μg mL^{-1}) significantly promotes the proliferation and mineralization of rat dental pulp stem cells *in vitro* (Ning *et al.* 2020). The antibody assay results revealed that the expression of TWEAK was downregulated at 72 h compared with 12 h post-treatment. TWEAK is reported to be a pro-inflammatory molecule that can promote the pro-inflammatory activities of other cytokines involved in local inflammatory responses, such as TNF-α, IL-1 and IL-6 (Liu *et al.* 2017). In addition, a sustained increase in the level of the anti-inflammatory cytokine IL-10 was observed at 72 h. Moreover, the level of Notch-2, an important regulator of stem cell fate, was significantly higher in the LPS-treated pulp at 72 h than in the control pulp (Mitsiadis *et al.* 2017a,b). The expression levels of TIMP-1 and β-NGF were decreased compared with those at 12 h but were still significantly higher than those in the normal pulp. Overall, at 72 h, the LPS-treated pulp was partially inflamed with emerging tissue repair events. Correspondingly, the levels of multiple anti-inflammatory or repair-enhancing factors were significantly upregulated, suggesting that the local microenvironment of the dental pulp might become more conducive to tissue repair.

The present study revealed that some inflammatory mediators were differentially expressed in certain states of pulpal inflammation, suggesting that these molecules might be possible biomarkers revealing the inflammatory conditions in the pulp. The two molecules with the highest fold-changes amongst the three groups were selected from 13 differential proteins as potential biomarkers for ELISA validation and further analysis. The levels of TIMP-1 were changed remarkably and positively correlated with the degree of pulpitis. However, the correlation between the levels of β-NGF and the inflammatory severity in pulp tissues was relatively weak. Therefore, it is speculated that TIMP-1 might be a more likely indicator of the pathological status of inflamed pulp.

In this study, the level of TIMP-1 significantly increased with the aggravation of pulpal inflammation. The results appear to be consistent with previous reports, in which TIMP-1 expression was found to be upregulated in human pulp tissues and dentinal fluids in teeth diagnosed with irreversible pulpitis (Mente *et al.* 2016, Brizuela *et al.* 2020). Spearman's correlation analysis revealed that the level of TIMP-1 in dental pulp tissues was strongly and positively correlated with the severity of pulpitis in the apical three-quarters of the pulp. Immunofluorescence staining further revealed that from 9 to 24 h, the expression of TIMP-1 in the apical three-quarters of the pulp was significantly higher than that in the control group. Several types of immune cells, such as neutrophils, monocytes and B cells, can secrete TIMP-1 into the extracellular matrix (Khokha *et al.* 2013, Wang *et al.* 2019). Upregulated TIMP-1 expression can activate fibroblasts, and in turn, activated fibroblasts further release more TIMP-1 into the extracellular matrix (Iredale *et al.* 2017). In addition, pro-inflammatory cytokines involved in pulpal responses to LPS, such as IL-1β and TNF-α, also upregulate TIMP-1 expression in dental fibroblasts (Lin *et al.* 2001). Therefore, activated dental pulp cells and immune cells may provide higher levels of TIMP-1 in pulpitis. TIMP-1 is traditionally thought to be a regulator of the extracellular matrix that functions by binding to active MMPs and inhibiting their proteolytic activity (Jackson *et al.* 2017). Thus, although PMNs accumulated at a moderate density in the apical three-quarters of the pulp during this period, the integrity of the pulp tissue was not irreversibly damaged by proteases released by immune cells (Farges *et al.* 2015). By inhibiting proteolytic degradation of the extracellular matrix by MMPs, TIMP-1 can also limit excessive aggregation of neutrophils to prevent the overproduction of reactive oxygen species and proteases, thus protecting pulp tissues from degradation (Kim *et al.* 2005). However, a recent study reported that a novel form of TIMP-1 expressed on the surface of activated PMNs could play a counterintuitive role in promoting PMN pericellular proteolysis by binding MMP-8 and MMP-9 to the PMN surface (Wang *et al.* 2019). Considering that the different forms of TIMP-1 (soluble and membrane-bound) lead to differences in biological function, the specific role of TIMP-1 in regulating the proteolytic

activity of MMPs in dental pulp tissues needs to be further studied.

Increasing evidence has revealed that TIMP-1 can act as a signalling molecule with cytokine-like activities, regulating cell proliferation, apoptosis, differentiation and angiogenesis in an MMP-independent manner (Khokha *et al.* 2013, Ries 2014). A previous study reported that TIMP-1 could activate normal granulocytes in patients with acute pyelonephritis. Moreover, TIMP-1 could specifically block the transmigration of granulocytes, protect them from apoptosis and increase the respiratory burst (Chromek *et al.* 2004). The regulatory effects of TIMP-1 on granulocytes might in part explain the positive correlation of TIMP-1 with inflammation intensity. Intriguingly, TIMP-1 plays a role in dental pulp repair in an MMP-independent manner. It has been reported that TIMP-1 can be expressed in dental pulp cells and significantly promote the proliferation and odontogenic differentiation of dental pulp cells (Okamoto *et al.* 2019). In addition, TIMP-1 has a function similar to that of mineral trioxide aggregate, inducing tertiary dentinogenesis in a pulp capping model (Okamoto *et al.* 2019). In this study, clear signs of tissue repair were observed in the pulp 72 h after LPS treatment; specifically, a slight layer of hard tissue deposition and dense fibre bundles surrounding the PMN halo were observed. The high expression of TIMP-1 in the inflamed pulp indicated that TIMP-1 might facilitate subsequent tissue repair events by promoting the proliferation of dental pulp stem cells and dentinogenesis. In general, TIMP-1 might play an important role in the response to dental pulp injury due to its multiple biological functions. Further investigations are required to elucidate the mechanisms by which TIMP-1 regulates dental defence and repair in pulpitis.

Previously, potential biomarkers for pulpitis were usually identified based on various pro-inflammatory cytokines that are responsible for the initiation and aggravation of inflammation (Rechenberg *et al.* 2016, Hirsch *et al.* 2017, Zanini *et al.* 2017). In teeth clinically diagnosed with irreversible symptomatic pulpitis, the expression of TNF-α, IL-1β, IL-6, IL-8 and MMP-9 was found to be significantly increased compared with that in control teeth (Abd-Elmeguid *et al.* 2013). However, due to a lack of histologic evidence, there are no data to generate a full assessment of the diagnostic efficacy of these markers. In fact, repair-related molecules involved in dental defence show notable changes with the progression of pulpal inflammation. The results of the present study reveal a strong correlation between the repair-enhancing molecule TIMP-1 and the severity of pulpitis. Previous studies have also found that the levels of several repair-enhancing molecules, such as osteocalcin, vascular endothelial growth factor and fibroblast growth factor, are significantly elevated in human teeth diagnosed with reversible pulpitis (Emilia & Neelakantan 2015). These studies have revealed that repair-related molecules may serve as a good basis for developing potential biomarkers to differentiate certain states of pulpal inflammation. In addition, these molecules may exhibit the potential to precisely indicate the healing potential of injured pulp and predict the prognosis of vital pulp therapy.

This study presented an inflammatory molecule profile based on histologic changes in pulpitis. However, there were some limitations to this study. The immune response in rat incisors could be different from that in mature human permanent teeth, as rodent incisors are continuously erupting teeth with a high healing capacity (Renard *et al.* 2016). Since LPS-induced pulpitis models mainly represent the progression of acute inflammation, in further studies, it will be necessary to establish a model that simulates natural infection, which could help explore the molecular profiles of chronic pulpitis, as it is the most common type of pulpal inflammation in clinical practice. Hence, considering the differences in the biological characteristics of human and rat teeth, clinical sampling is required to explore the diagnostic potential of TIMP-1 and other potential biomarkers for pulpitis. Moreover, the screening of larger numbers of inflammatory mediators is encouraged to identify combined biomarkers, which could improve the precision of diagnostic performance.

Conclusion

This study provides a molecular profile of LPS-induced pulpitis developed on the basis of histologic changes in a rat model. The results revealed that TIMP-1 had a strong positive correlation with the severity of dental pulp inflammation, verifying the feasibility of applying biomarkers to identify specific pathological conditions in pulpitis.

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Conflict of interest

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Supplementary Material