Pulpal Tissue Inflammatory Reactions after Experimental Pulpal Exposure in Mice

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Abstract

Introduction: The purpose of this study was to establish a stable experimental mice pulp inflammatory model and to evaluate inflammatory reactions of pulp tissue after pulp exposure. Methods: Pulpal inflammation was induced in 80 C57BL/6 mice by occlusal exposure of the pulp of the maxillary first molar. The mice were sacrificed randomly at 0, 1, 6, 12, 24, 48, and 72 hours after pulp exposure. Mice without pulp exposure served as controls. Results: As the duration of pulp exposure increases, the inflammatory reaction is exacerbated. Within 6 to 12 hours after pulp exposure, pulp tissues experienced red blood cell extravasation to the destruction of the odontoblast layer. After 24 hours, necrosis was observed in the pulp tissue; until 72 hours, necrosis spread to the whole coronal pulp tissue, and a large number of inflammatory cells were found in the radicular pulp tissue. The results of histomorphometric scores of inflammation in vivo share the same trend; samples from the 72-hour group possessed the highest score followed by samples from other groups (P < .01). The expression levels of inflammatory cytokines increased over the 72 hours, and there was a high rate of inflammatory cytokine expression at 6 and 12 hours after pulp exposure. Conclusions: Our study represents a stable mice model for studying pulp inflammation in vivo. Mouse pulp inflammation progresses rapidly, with dramatic changes evident in just a few hours. (J Endod 2017;43:90–95)

Key Words

Animal model, histomorphologic evaluation, pulp inflammation

Inflammation is one of the most common oral diseases and is mainly caused by bacterial infection of the dental pulp. The introduction of bacteria into the pulp can easily result in irreversible pulpitis that impairs the spontaneous healing of the pulp tissue, ultimately resulting in necrosis and death of the pulp tissue. During pulp inflammation, dental pulp cells secrete a variety of inflammatory cytokines that participate in the immune response and cause pain (1), leading to periapical inflammation, which exacerbates a patient’s suffering.

In recent years, researchers have focused on studying the molecular mechanism of pulpitis. However, the signaling pathways of pulpitis development have not been clarified; therefore, a reasonable and stable animal model is needed to study the development mechanism of pulpitis.

Many investigators have induced periapical and pulp lesions in mammals in vivo (2–4). To date, these investigations have focused on the histologic description of periapical inflammation in mice but not on a description of pulp inflammation.

Also, most of these studies involved large animals, such as monkeys, dogs, and pigs, which have higher similarity to humans regarding teeth anatomy, evolution, and development, or animals that are easier to operate on such as rabbits and rats. Compared with the aforementioned animals, mouse models of pulp inflammation have distinct advantages. For instance, sample sizes can be larger, and models using knockout mice are easy to create.

Therefore, in the present study, we induced pulp inflammation of mice by pulp exposure at 0, 1, 6, 12, 24, 48, and 72 hours; we used inflammatory parameters and scores for evaluation and observed changes in inflammatory cytokine expression in order to create a convenient model for studying the pathogenesis of pulp inflammation in vivo.

Material and Methods

Induction of Pulpal Lesion

All animal studies were approved and supervised by the Ethics Committee of Affiliated Stomatology Hospital, Guangzhou Medical University, Guangzhou. Eighty C57BL/6 mice (8–10 weeks old) weighing 20–30 g were purchased from the Medical Laboratory Animal Center of Guangdong Province, China, and randomly divided into 8 groups. Seventy mice were anesthetized by an intraperitoneal injection of pentobarbital sodium (50 mg/kg), and a cavity was prepared with a #1/4 dental round bur on the occlusal surface of the bilateral upper first molars (class 1 cavity) under a surgical microscope (×40). The upper first molar was drilled (medium speed with cooling system) until the pulp was visible through the transparency of the dentin floor of the cavity. Pulp was subsequently exposed using an endodontic hand file (0.15-mm diameter tip, 2% taper, 21 mm). The exposed preparation cavity (Fig. 1A–C) was left open to the oral environment. Ten animals without exposed pulp served as the controls.
Sample Preparation

Ten mice in each group were sacrificed at 0, 1, 6, 12, 24, 48, and 72 hours after pulpal exposure. Five maxillae were dissected free of soft tissues, fixed with 4% paraformaldehyde at 4°C for 48 hours, and then imaged using micro–computed tomographic (micro-CT) imaging. After micro-CT image acquisition, these samples were prepared for histologic analysis. The other 5 maxillae (including 10 teeth that were extracted from maxillae) were used for analysis of the inflammatory cytokines.

Micro-CT Analysis

After being washed in running water, each maxilla with the tooth was dried, mounted on a custom attachment, and scanned in a micro-CT scanner (SkyScan 1172; Bruker-microCT, Kontich, Belgium) operated at 60 kV and 100 mA (0.5-mm Al filter). The scanning was performed by rotating the mounted sample 180° around the vertical axis, with a rotation step of 0.4°. The cross-sectional pixel size and intersection distance were both 9.93 mm. Images were reconstructed (NRecon v.1.6.3, Bruker-microCT) to obtain axial cross sections of each specimen’s inner structure. DataViewer v.1.5.1.10 software (Bruker-microCT) was used for 2-dimensional evaluation of the diameter of the pulpal exposure site.

Histopathologic Analysis

The maxilla with the tooth was rinsed and decalcified with 10% EDTA for 6 weeks, dehydrated, and embedded in paraffin; 4-μm thick serial sections were cut in the mesiodistal direction and used for histologic analyses.

Qualitative and quantitative histopathologic analyses were performed on hematoxylin-eosin–stained specimens using the bright-field mode of a fluorescence microscope (Leica/DM4000 B; Leica Microsystems, Heidelberg, Germany). We chose 5 samples at control 0, 1, 6, 12, 24, 48, and 72 hours after pulp exposure, respectively. During each time point, each sample was selected at a 5–6 high microscope field of vision, which has the maximum area of coronal pulp tissue for quantitative analysis. An experienced examiner blinded to the groups conducted all analyses.

The histomorphologic parameters used in this study (Table 1) were based on criteria previously described (5, 6); each parameter was scored 1 to 4 (with 1 being the best result and 4 being the most severe). The study-wide null hypothesis was that the distributions of histologic findings would be the same among the 8 experimental groups for all 5 histomorphologic parameters. To reduce the study-wide type I error rates, multigroup comparisons were performed using a Bonferroni correction alpha of $P = .0018$. For each histomorphologic parameter, a null hypothesis of equal distributions among the 8 groups was tested using the Kruskal-Wallis H test for comparison of multiple independent samples of frequency table data. If $P$ was <.05 for differences between the 8 groups, then Mann-Whitney U tests were performed to identify which groups were significantly different from each other.

Real-Time Polymerase Chain Reaction

Teeth were collected from groups of 5 animals at intervals of 0, 1, 6, 12, 24, 48, and 72 hours after cavity preparation. Teeth were also collected from control animals. After removing surrounding tissue, the teeth were stored in liquid nitrogen until used.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Location of inflammatory cells</th>
<th>Intensity of the inflammatory infiltrate</th>
<th>Inflammatory edema</th>
<th>Vascular leakage</th>
<th>Extension of pulp necrosis (coronal pulp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scores</td>
<td>Absent (score 1)</td>
<td>0–20 inflammatory cells (score 1)</td>
<td>Absent (score 1)</td>
<td>Absent (score 1)</td>
<td>1%–10% necrotic area (score 2)</td>
</tr>
<tr>
<td></td>
<td>Restricted to the exposed site (score 2)</td>
<td>21–40 inflammatory cells (score 2)</td>
<td>Present (score 2)</td>
<td></td>
<td>11%–20% necrotic area (score 3)</td>
</tr>
<tr>
<td></td>
<td>Pervaded in the entire coronal pulp (score 4)</td>
<td>41–80 inflammatory cells (score 3)</td>
<td>Present (score 2)</td>
<td></td>
<td>Over 20% necrotic area (score 4)</td>
</tr>
</tbody>
</table>

TABLE 1. Parameters and Scores Used for Evaluation
The total cellular RNA was obtained from pulp tissues using a MiniBEST Universal RNA Extraction kit (TaKaRa Biotechnology Co, Dalian, China) according to the manufacturer’s recommendations. Reverse transcription was performed using PrimeScript RT Master Mix (TaKaRa) according to the manufacturer’s protocol. Real-time polymerase chain reaction (PCR) was performed with a CFX96 Real-Time PCR Detection System using 25 μL SYBR Premix Ex Taq II solution (TaKaRa). Primers were designed by GeneCopoeia, Inc, Guangzhou, China. The catalog numbers for glyceraldehyde-3-phosphate dehydrogenase, interleukin (IL)-1β, IL-6, and tumor necrosis factor alpha (TNF-α) were MQP027158, MQP027422, MQP036632, and MQP031019, respectively. Reaction mixtures were denatured for 30 seconds at 95°C and subjected to 40 cycles of PCR comprising denaturation for 5 seconds at 95°C and annealing for 30 seconds at 60°C. The relative messenger RNA expression was normalized to internal control glyceraldehyde-3-phosphate dehydrogenase. The ΔΔ Ct method was performed to analyze mRNA expression levels. Statistical analyses were performed using 1-way analysis of variance followed by the Student-Newman-Keul test with SPSS 13.0 software (SPSS Inc, Chicago, IL). P < .05 was considered to indicate significant differences. All data were presented as the mean ± standard deviation.

Results

Micro-CT Observation

Micro-CT images showed the pulp and periapical area. The size of the pulpal exposure area stayed approximately the same, without crown or root fracture in the sagittal, transverse, or coronal directions (Fig. 1D–F). At 72 hours after pulpal exposure, no periapical shadows or alveolar bone resorption were observed.

Histopathologic Observation

Sections with a complete root canal, which included the apical foramen, were selected for measurement to ensure that each section represented the largest pulpal lesion area. The pulpal region was intact, and no inflammatory cell infiltration was observed in the control pulpal tissues; odontoblasts were arranged regularly inside the dentin (Fig. 2A). At 0 hours, there was little change compared with samples from the control group (Fig. 2B). At 1 hour after pulpal exposure, some inflammatory cells appeared in the exposed site (Fig. 2C). At 6 hours, a slight infiltration of inflammatory cells was found. Pulpal angiectasis, hyperemia, and substantial red blood cell extravasation were observed throughout the whole coronal pulpal tissues (Fig. 2D). At

Figure 2. Histologic results of mouse pulpal regions from control to 72 hours. As the duration of pulpal exposure increases, the inflammatory reaction is exacerbated. A1, B1, C1, C2, D1, E1, F1, G1, G2, H1, and H2 were presented with the high magnification of black frame in A–H. The arrow shows the red blood cell extravasation. The triangle shows necrosis tissues (scale bars = 50 μm).
12 hours, signs of mild infiltration of acute inflammatory cells appeared, and slight damage to the odontoblast layer was observed near the exposed pulp (Fig. 2E). At 24 hours, necrosis was observed in the pulpal tissue adjacent to the exposed pulp, and severe infiltration of inflammatory cells was confined to one half of the coronal pulpal tissues. The odontoblast layer was disordered (Fig. 2F). At 48 hours, one fifth of the coronal pulpal tissues had undergone necrosis, and chronic inflammatory cell infiltration was evident. Most odontoblasts were missing (Fig. 2G). At 72 hours, odontoblasts were missing, chronic infiltration of inflammatory cells around all pulpal tissues and over one fifth of the necrotic area was observed throughout the whole coronal pulpal tissue, and a large number of inflammatory cells were found in the radicular pulpal tissue (Fig. 2H).

Quantification of Histopathologic Findings

The quantification of histopathologic findings was shown in Table 2. Data were analyzed using SPSS 13.0 software (SPSS Inc, Chicago, IL). There were significant differences found among the 8 groups \((P < .0018)\), and 72 hours had the highest scores in the 5 parameters. The inflammatory reaction extended to all of the coronal pulp in all of the samples after 6 to 72 hours of exposure, possessing higher scores than samples at 0 and 1 hour \((P < .01)\). Interestingly, massive infiltration of inflammatory cells was noted soon after the dental pulp was exposed to the oral environment; significant differences were observed between the 1-hour and control groups \((P = .000)\) compared with the control. As the duration of pulp exposure increased, the infiltration of inflammatory cells increased dramatically at each time point \((P < .01)\) compared with the control. At 6, 12, 24, 48, and 72 hours, inflammatory edema and vascular leakage were observed in all of the samples, with scores being statistically different from samples from the control, 0-hour, and 1-hour groups \((P = .001)\). Scores for pulp necrosis also exhibited an increasing trend; a large necrotic area was observed in the coronal pulp in the 72-hour group, which possessed the highest score; and the scores in the 24-hour, 48-hour, and 72-hour groups were higher than the control, 0-hour, 1-hour, and 6-hour groups \((P = .000)\).

**IL-1β, IL-6, and TNF-α mRNA Expression in Pupal Inflammation**

The expression of IL-1β, IL-6, and TNF-α was analyzed by the detection of the relevant mRNA at each time point. As seen in Figure 3, IL-1β mRNAs were detected at 6 hours after bacterial infection, reached their maximum levels at 12 hours, and then decreased, with a general upward trend over the remaining time points compared with the control group. IL-6 mRNAs were detected at 1 hour after bacterial infection and then increased dramatically to their maximum levels at 6 hours. IL-6 mRNA levels decreased after 12 hours but exhibited a general upward trend over the remaining time points compared with the control group. TNF-α mRNAs were detected at 1 hour after bacterial infection. Expression levels of TNF-α fluctuated between time points but peaked at 12 and 48 hours, with relative fold changes of 2.13 and 2.55, respectively.

**Discussion**

Pulpitis is the most common inflammatory disease in humans. Rapid development of inflammation is characteristic of pulpitis, with dramatic changes noticeable in just a few hours.

In agreement with previous studies \((7, 8)\), pulpal inflammation could be reproducibly induced in mice by pulpal exposure and subsequent bacterial infection from the oral environment. Pulpal exposure and injury serve as a stable experimental model for
observing the progress of pulp inflammation. In our study, pulp exposure was created mechanically using an endodontic hand file (0.15-mm-diameter tip, 2% taper, 21 mm) as described by Frozoni et al (9); the area of the pulpal exposure across multiple samples can be kept approximately the same (about 0.15 mm), which helps keep the development of inflammation as consistent as possible. Compared with our mouse model of pupal inflammation, the inflammation reaction progresses rapidly in rats. In earlier studies with rats, however, larger pulp lesions were used or lesions were not created with an endodontic hand file (10, 11).

The results of hematoxylin-eosin staining revealed that the pulp, without enamel and dentin protection, is vulnerable to bacterial invasion, which leads to dramatic inflammation of the pulp in just a few hours. The results were consistent with those of a previous study before (12). Regarding the histopathological changes of pupal inflammation, mice and rat models of pupal inflammation development exhibit the same trends, which include light, medium, and severe inflammation and tissue edema, exudation, and necrosis. As the duration of pulpal exposure increases, the severity of pupal inflammation tends to increase; however, pupal inflammation in the mouse model occurred more rapidly than in the rat model.

The results of histomorphologic scores have the same trend. At 1 hour after pulpal exposure, numerous inflammatory cells are restricted to the pulpal lesion, which may be associated with an acute inflammatory reaction caused by mechanical stimulation. As the duration of pulpal exposure increases, the inflammatory reaction is exacerbated along with the invasion of inflammatory cells and subsequent tissue disorder.

The expression levels of inflammatory cytokines are important indices of pupal inflammation. During bacterial infection, Th1 and Th2 responses are believed to be critical to inflammation. Therefore, understanding the status of the Th1 and Th2 responses should provide insight into the pathogenesis of pupal inflammation (13, 14). The expression levels of Th1 cytokines (eg, TNF-α) and Th2 cytokines (eg, IL-1β and IL-6) change in pupal inflammation tissues. The gene expression levels of TNF-α fluctuated between time points, with peaks of 2.13- and 2.55-fold, respectively, at 12 and 48 hours after pulpal exposure. Compared with the control group, gene expression levels of IL-1β and IL-6 began to increase at 6 hours after pulpal exposure. In particular, gene expression levels of IL-6 increased dramatically to their peak value. These results suggest that Th1 and Th2 responses are active in the development of pupal inflammation and that the Th1 response plays a leading role at 6 hours after pulpal exposure.

Elsalhy et al (15) regard cytokines as diagnostic markers of pulpal inflammation. They obtained blood from normal teeth, asymptomatic caries-exposed pulps, and teeth with irreversible pulpitis and subsequently measured and compared the levels of IL-2, IL-6, IL-8, IL-10, TNF-α, and interferon gamma in pulpal blood using enzyme-linked immunosorbent assay. They found that IL-2 and IL-10 levels as well as IL-6/IL-10 and IL-8/IL-10 ratios were significantly higher in irreversible pulpitis compared with caries-exposed and normal teeth. To a certain extent, the changes of cytokine levels can reflect the severity of inflammation. Between 1 and 6 hours, there are obvious changes in the response of pulp tissues to pupal inflammation, and the expression levels of inflammatory cytokines were significantly higher at 6 and 12 hours compared with the control group. Thus, dramatic changes in the pulp tissue of mice take place during the first 12 hours of pulpal exposure.

The main function of IL-1β is to promote the activation of mononuclear macrophages, thereby causing changes in acute response–related proteins (16). The function of TNF-α is to promote the migration of neutrophils and monocytes via chemotaxis, leading the 2 types of cell activation and degranulation and releasing inflammatory mediators (17–19). IL-6 is a multifunctional cytokine, which is produced by Th2 cells, monocytes, vascular endothelial cells, and fibroblasts. Its inflammatory response is well verified; however, recently, it has also been shown to be an anti-inflammatory cytokine (20, 21). In the early stages of pupal inflammation, pulp tissue produces a series of cytokines that take part in repair responses to resist the invasion of foreign matter (22). IL-6 may take part in such responses, which could explain why the expression levels of IL-6 are so high at 6 hours after pulpal exposure. This requires further verification.

Figure 3. The relative fold change of IL-1β, IL-6, and TNF-α of pupal inflammation in a mouse at each time point. There were significant differences between each group (*P < .05). Data are presented as mean ± standard deviation.
In this study, a convenient mouse model was established for studying the pathogenesis of pulp inflammation in vitro. Our study provides a novel opportunity to enable the use of genetically modified animals to explore cellular and molecular processes during pulp inflammation; a number of factors that were expressed in the early pulp inflammation may be studied (2.5–25).

In this study, we created a stable experimental model for observing the progress of pulp inflammation. In the early stages of pulp inflammation, pulp tissue exhibits obvious changes in just a few hours. The expression levels of inflammatory cytokines were significantly higher at 6 and 12 hours. At 72 hours after pulp exposure, necrosis was observed throughout the pulp tissue. Our study is a valuable reference for further research into the molecular mechanism of pulp inflammation.

Acknowledgments

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

References
