Research Note

High-cholesterol Condition Promotes Apical Periodontitis and Bone Resorption in Rats

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Abstract: The objective of this study is to investigate the influence of high cholesterol condition on apical periodontitis and bone resorption in rats. Six-week-old rats were fed a high cholesterol diet (HCD) and compared to normal diet (ND) rats. The pulp of the first maxillary molar was exposed and apical periodontitis was generated experimentally, and bone resorption was evaluated. At 6 weeks after pulp exposure, apical periodontitis were noted histologically and radial bone resorption was observed in the HCD rats. TRAP staining revealed the number of osteoclasts in the HCD group to be significantly higher than that in ND rats (p<0.05). Significant differences in mRNA expression in toll-like receptor 2 (TLR2) and RANKL were detected in HCD rats. These results suggest that a high-cholesterol condition promotes apical periodontitis and bone resorption via expression of TLR2 and RANKL in rats.

Key words: High cholesterol, Apical periodontitis, Osteoclastogenesis, Bone resorption, RANKL, TLR2

Introduction

Many diseases in dentistry, such as dental caries, apical periodontitis and periodontal disease, result from endogenous infections. Apical periodontitis is frequently caused by microorganisms within the root canal1)2), and bacterial presence has also been associated with intractable apical periodontitis. Inflammatory cell infiltration in the apical periodontal ligament and bone resorption in the apical alveolar bone occur as defensive reactions of host. Therefore, root canal treatment involves the mechanical removal of infected dentin from the root canal walls and chemically disinfecting the root canal systems.

Correlation between oral infections and systemic conditions, including cardiovascular disease, diabetes mellitus, and preterm low birth weight, has been suggested3). In the last two decades, “periodontal medicine” has developed as a distinct area that focuses on the relationship between periodontal disease and systemic diseases4). Recently, apical periodontitis is not thought to be only a local phenomenon, and the medical and dental scientific communities have analyzed the possible correlation between apical periodontitis and systemic health5). Increasing numbers of reports describing the association between periapical inflammation and systemic diseases such as diabetes mellitus, hypertension and non-alcoholic fatty liver disease (NAFLD), and therefore, “periodontal medicine” has developed. On the other hand, the impact of systemic diseases and some general habits on pulp and periapical health remains unknown and needs to be further investigated.

Hyperlipemia, a major lifestyle disease generated by eating habit, is regarded as a risk factor for stroke and myocardial infarction due to atherosclerosis, and 78 million US adults9) have a high cholesterol level. Having high blood cholesterol increases the risk of heart disease, the leading cause of death in the US, and it is now a global health issue6). Recent studies reported that hyperlipemia is associated with not only atherosclerosis, but also an increase in inflammatory cytokines induced by Porphyromonas gingivalis and alveolar bone loss in periodontitis5)6)7). Furthermore, the level of inflammatory cytokines induced by P. gingivalis increases in mice fed a high fat diet8). Moreover, lipids and lipoproteins are involved in the increase in inflammatory markers and lesion size in patients with chronic apical periodontitis10). However, the detailed mechanism of bone resorption in hyperlipemia remains unknown. Of note, one study found that osteoclast formation, survival, and morphology are highly dependent on exogenous cholesterol/ lipoproteins, and the life span of osteoclasts becomes shorter under low-cholesterol conditions11). However, there is little information as to whether high-cholesterol conditions affect bone resorption in apical periodontitis.

In the present study, to understand the relationship between high cholesterol and apical periodontitis, we investigated bone resorption of experimentally induced apical periodontitis in rats fed a high cholesterol diet.

Materials and Methods

Animal experiments were carried out in accordance with the Guidelines for the Treatment of Animals at Tokyo Dental College (approval number: 302101).

Animals and experimental design

Six-week-old male Wistar rats (n=18) were purchased from Sankyo Labo Service Corporation (Tokyo, Japan) and used in this study. The rats were divided into two groups; one group was fed a normal diet (ND) (MF; Oriental Yeast Corporation, Tokyo, Japan) and another was fed a high cholesterol diet (HCD) (MF with 1% cholesterol, 1% cholic acid, and 5% lard; Oriental Yeast Corporation) by reference to the method by Morishita et al. (1986)10) and Yamamoto et al. (2010)11). The rats were housed in a temperature-, humidity-, and light-controlled room, and fed...
The rats in control group were fed ND and the rats in experimental group were fed HCD throughout the study. Feeding ND or HCD was started from 2 weeks prior to pulp exposure. Apical periodontitis was induced according to the method by Azuma et al. (2018) with modification. The rats were anesthetized with pentobarbital sodium (Somnopentyl®, Kyoritsuseiyaku Corporation, Tokyo, Japan), and the pulp chamber in the maxillary right first molar (M1) was opened using a steel round bar (Time 0), and the coronal pulp tissue was exposed to the oral cavity and was not sealed afterward. Apical area of mesial root in the maxillary M1 was evaluated at time points.

Measurement of total cholesterol
For evaluation of blood cholesterol, blood was intravenously extracted from the tail end every other week (n=5 in each group). Blood samples were centrifuged and serum was obtained. Total cholesterol was measured using LabAssay™ Cholesterol (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

Micro-computed X-ray tomography (micro CT) analysis
Micro-CT scans of maxillary M1 were performed at the molar level using an R-mCT scanner (Rigaku Corporation, Tokyo, Japan) with the following imaging conditions; tube voltage 90 kV, tube current 100 μA at 2 and 4 weeks after pulp exposure. The obtained digital image was processed to reconstruct three-dimensional (3D) images using reconstruction software (TRI/3D-BON; Ratoc System Engineering Co. Ltd., Tokyo, Japan). The voxel size was 50 × 50 × 50 μm³. The raw data were reconstructed as Tag Image File Format (TIFF) images. Volume in both groups was measured at 4 weeks (n=5 in each group).

Tissue preparation
At 6 weeks after opening pulp chamber, the rats were sacrificed and transcardially perfused with 4% paraformaldehyde - 0.1 M phosphate buffer (PFA; pH 7.4) for fixation (n=6 in each group). Dissected maxilla and liver were immersed for 24 h in the same fixative. The maxilla was decalcified for 4 weeks at 4°C in 10% ethylenediaminetetraacetic acid (EDTA) containing 7% sucrose. The tissues were embedded in paraffin and then cut into 4-μm-thick sections. The livers were washed in 0.01 M phosphate-buffered saline (PBS, pH 7.4) after immersion and embedded in OCT compound (Sakura-Finetek Japan Co. Ltd., Tokyo, Japan), and 10-μm-thick frozen sections were prepared. Both sections were stained with hematoxylin-eosin (H-E) for routine histological observation.

Tartrate-resistant acid phosphatase (TRAP) staining
TRAP staining was carried out using the TRAP/ALP Stain kit (FUJIFILM Wako Pure Chemical Corporation). After the sections were deparaffinized, they were incubated in tartaric acid solution and acid phosphatase substrate for 30 min, and nuclei were stained. The length of the bone surface in periapical lesions was measured, and TRAP-positive cells per mm on the bone surface were counted (n=4 in each group).

Quantitative real-time polymerase chain reaction (qRT-PCR)
Total RNA was extracted from apical area of mesial root in maxillary M1 (n=3 in each group) using a Teflon-glass homogenizer and TRIzol® reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA) and subsequently dissolved in RNase-free water (Takara Bio Inc., Otsu, Japan). Total RNA was added to the reaction mixture and transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The TaqMan MGB probes for TLR2 (Rn02133647_s1), TLR4 (Rn00569848_m1), RANKL (Rn00589289_m1), OPG (Rn00563499_m1), and 18S rRNA as an endogenous control were purchased from Applied Biosystems, and qRT-PCR was performed using TaqMan MGB probes (Applied Biosystems), the TaqMan Fast Universal PCR Master Mix (Applied Biosystems), and 7500 Fast Real-Time PCR System (denature 95°C, 3 sec; annealing/extension 60°C, 30 sec, 40 cycles, Applied Biosystems). The relative gene expression was estimated using the ΔΔCt method.

Figure 1. Total cholesterol. The serum level of total cholesterol in the ND and HCD groups increased with time, and fold changes ranged from 2.5 to 3.5 between groups, being significantly different (n=5; *p<0.05).

Figure 2. Histological assessment of the liver. Hepatocytes were solid and had a pink cytoplasm in the ND-fed rats (a), whereas they were clear in the HCD-fed rats, reflecting lipid deposition (b and c). c: higher magnification. Bars = 200 μm (a, b), Bar = 50 μm (c)
Statistical analysis

Data are expressed as the mean ± standard deviation (SD) or standard error (SE). Significant differences between two groups were evaluated using Mann-Whitney’s U-test ($P<0.05$).

Results

Total cholesterol and histological assessment of the liver

Rats had been fed with the ND or HCD, and the value of total cholesterol from the serum was measured. The level in the ND group at 2 weeks was 51.2±13.0 mg/dl, that at 4 weeks was 53.2±16.0 mg/dl, and that at 6 weeks was 45.2±8.1 mg/dl. In the HD group, the size of cholesterol increased with time; 2 weeks 126.8±58.1 mg/dl, 4 weeks 142.6±54.8 mg/dl, 6 weeks 172.0±82.3 mg/dl. Fold changes ranged from 2.5 to 3.5 between the two groups and were significant ($P<0.05$) (Fig. 1).

The cytoplasm of the hepatocytes was eosinophilic and deposits were not observed in the ND-fed rats (Fig. 2a). On the other hand, it was clear in the HCD-fed rats, resembling lipid deposition (Fig. 2b). The hepatocytes of HCD-fed rats showed small granules and clear lipid droplets (Fig. 2c). Necrosis, fibrosis and inflammatory cell infiltration were not observed in the HCD group at 6 weeks (data not shown).

Based on the serum total cholesterol and histological findings of the liver, we used this animal model in this study.

Micro-CT findings of apical lesion

Micro-CT analysis showed apical radiolucency was detected in the both ND (Fig. 3a) and HCD (Fig. 3b) groups at 2 weeks after pulp exposure, and the lesions became larger at 4 (Fig. 3c, d). In HCD group, the size was larger and destruction of apical bone was detected, and the lesion expanded in maxillary sinus at 4 weeks (Fig. 3d). The 2-weeks (Fig. 3a, b) and 4-weeks (Fig. 3c, d) images were colored in red and green, respectively and merged (Fig. 3e, f). The merged images showed radiolucency in HCD group (f) was larger than that in ND group (e).

Histological assessment of the apical lesions

At 6 weeks after pulp chamber opening, histological analysis about apical area of mesial root in maxillary M1 was carried out. Abscess formation was histologically observed at apical regions in both groups. An increase in the number of fibroblasts, and inflammatory cell infiltration composed of macrophages and lymphocytes were observed around the abscess area. Periapical bone resorption was also noted and the surface of the bone was smooth in the ND group (Fig. 5a), whereas the bone surface was radially resorbed in the HCD group (Fig. 5b).

On TRAP staining, TRAP-positive cells resembling osteoclasts with a single or multiple nuclei were observed at the bone surface of the apical lesion in the ND and HCD groups (Fig. 5c, d). The number of TRAP-positive cells at the bone surface of an apical lesion was 1.88±0.52 per mm in the ND group and 3.00±0.38 per mm in the HCD group. There was a significant difference between two groups ($P<0.05$) (Fig. 6).

mRNA expression of RANKL, OPG, TLR2, and TLR4

Expression of RANKL, OPG, TLR2, and TLR4 was evaluated by qRT-PCR. The expression of RANKL and TLR2 expression levels in the HCD group were significantly higher than those in the ND group (4.0-fold

Figure 3. Micro-CT images. Micro-CT analysis showed apical radiolucency was detected in the both ND (a) and HCD (b) groups at 2 weeks after pulp exposure, and the lesions became larger at 4 (c, d). In HCD group, the size was larger and destruction of apical bone was detected, and the lesion expanded in maxillary sinus at 4 weeks (d). The 2-weeks (a, b) and 4-weeks (c, d) images were colored in red and green, respectively and merged (e, f). The merged images showed radiolucency in HCD group (f) was larger than that in ND group (e).

Figure 4. Volume of bone resorption. Volume of bone resorption in HCD was significantly larger than that in ND group ($P<0.05$).
and 3.0-fold, respectively, $P<0.05$). However, we could not find statistical differences in the expression of OPG or TLR4 between the ND and HCD groups (Fig. 7).

**Discussion**

**Rat model of high cholesterol and apical periodontitis**

Animal models are necessary to investigate whether high cholesterol affects periapical lesions *in vivo*. Previous studies reported the generation of high-cholesterol conditions by diet \(^{13, 16, 17, 19}\). Earlier studies employed diets containing a high concentration of lard \(^{20, 21}\), but our preliminary study revealed no significant difference in the serum cholesterol or triglyceride level compared with a normal diet (data not shown). On the other hand, Yamamoto *et al.* \(^{17}\) generated high cholesterol model rats using a 1% cholesterol diet and induced larger hepatic damage by LPS stimuli. In the present study, model rats were generated by mixing 1% cholesterol, 1% cholic acid, and 5% lard according to the methods by Morishita *et al.* \(^{16}\). As a result, the total blood cholesterol level in the HCD group became higher than that in the ND group 2 weeks after feeding. Furthermore, fatty liver was confirmed histologically. Therefore, we employed these rats as a model of high cholesterol.

**Bacterial infection, receptors of bacteria and bone resorption related to high cholesterol**

TLR2 and TLR4 are receptors of Gram-positive and Gram-negative bacteria, respectively, and inflammatory cytokines, such as IL-1, IL-6, and TNF-alpha, are produced via NF-κB signals. The expression of inflammatory cytokines, such as IL-1 and IL-6, is involved in bone resorption at apical periodontitis \(^{22}\). Therefore, TLR2 and TLR4 are keys for...
cytokine production. Bacterial infection induced severe inflammation under fatty diet conditions due to the induction of higher expression of TLR2 and TLR413, 23). In the present study, the expression of TLR2 and RANKL in ND group was lower than that in HCD group after pulp exposure. The results imply that high-cholesterol conditions promote expression of TLR2 and RANKL signals.

On the other hand, there was no significant difference in TLR4 expression in this study. TLR4 increased when Gram-negative bacteria infected, but it was considered that upregulation did not occur because no bacterial infection was caused and SPF mice were used in this study. Further studies are required to confirm the mechanism.

**High cholesterol and osteoclastogenesis**

Previous reports found that high-cholesterol conditions affect alveolar bone resorption in periodontal disease12, 24-26). Both RANKL and OPG are proteins involved in the differentiation of monocyte stem cells into osteoclasts27). However, the mechanism of bone resorption via the RANKL pathway under high-cholesterol conditions remains unclear. Rubin et al.28) reported that cholesterol-controlled expression of RANKL via H-Ras is related to the activity of osteoclasts and that RANKL expression is inhibited by H-Ras-GTPase. RANKL is induced by inflammatory cytokines, such as IL-6, and promotes osteoclastogenesis29). Our micro-CT analysis revealed larger apical lesions in the HCD group than those in the ND group. Furthermore, the number of TRAP-positive cells resembling osteoclasts in the HCD group was higher than that in the ND group. The results of this study suggest that high-cholesterol conditions induce osteoclastogenesis.

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**Conflict of Interest**

The authors have declared that no conflict of interest (COI) exists.

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