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Effects of topical application of lipopolysaccharide and proteases on hepatic injury induced by high-cholesterol diet in rats

T. Yamamoto, T. Tomofuji, N. Tamaki, D. Ekuni, T. Azuma, T. Sanbe

Department of Preventive Dentistry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Shikata-cho, Okayama, Japan

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Background and Objective: Topical application of lipopolysaccharide and proteases to the gingival sulcus induced not only periodontal inflammation but also detectable liver changes in rats fed a normal diet. However, these changes in the liver were not sufficient to induce pathological consequences. The purpose of the present study was to investigate whether gingival inflammation-induced liver change would have more dramatic pathological consequences in rats fed a high-cholesterol diet compared with the effect of the high-cholesterol diet alone.

Material and Methods: Twenty-four male Wistar rats were divided into four groups. During an 8 week experimental period, two groups were fed a normal diet and the other two were fed a high-cholesterol diet containing 1% cholesterol (w/w) and 0.5% cholic acid (w/w). Four weeks prior to the end of the experimental period, one of each of the dietary groups received daily topical application of lipopolysaccharide and proteases to the gingival sulcus, while the other was treated with pyrogen-free water.

Results: In the rats without application of lipopolysaccharide and proteases, the serum level of hexanoyl-lysine, scores of steatosis and inflammation, and concentration of 8-hydroxydeoxyguanosine in liver of rats fed a high-cholesterol diet were higher than in those fed a normal diet. In rats fed a high-cholesterol diet, the scores of steatosis and inflammation and the concentration of 8-hydroxydeoxyguanosine in the liver of rats with application of lipopolysaccharide and proteases were higher than in those without.

Conclusion: In a rat model, application of lipopolysaccharide and proteases to the gingival sulcus augmented the effect of a high-cholesterol diet on steatosis, inflammation and oxidative damage in the liver.

Tatsuo Yamamoto PhD, DDS, Department of Preventive Dentistry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8525, Japan
Tel: +81 86 235 6711
Fax: +81 86 235 6714
e-mail: tatsuo@md.okayama-u.ac.jp

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Periodontitis is the inflammation of supporting structures of the tooth caused by chronic bacterial infection (1). Studies have suggested that periodontitis is a risk factor for systemic diseases, including diabetes mellitus (2), hyperlipidemia (3) and coronary heart diseases (4). The mechanisms by which periodontitis increases the likelihood of these systemic diseases have not been clearly defined, but the prerequisite is believed to be host response to long-term systemic exposure to bacterial pathogens [i.e. lipopolysaccharide (LPS) and proteases]. In spite

of numerous reports on the relationship between periodontitis and systemic diseases, little attention has been paid to the effects of periodontal infection on the liver, which plays an important role in glucose and lipid metabolism.

Epidemiological studies demonstrated an association between periodontitis and liver diseases by showing that the incidence of periodontitis increased with elevated serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and cholinesterase, and an AST-to-ALT ratio of less than one (5). In our previous study (6), chronic application of LPS and proteases to the gingival sulcus induced non-alcoholic fatty liver disease-like lesion in rats, with increasing periodontal inflammation and serum level of oxidative stress. The study also suggested that systemic oxidative stress, induced by application of a combination of LPS and proteases to the gingival sulcus, plays a central role in the initiation of a non-alcoholic fatty liver disease-like lesion. These findings indicate that periodontitis has an effect not only on diabetes mellitus and coronary heart diseases but also on liver disease. However, in the rat model of periodontal disease, serum levels of ALT were not increased (6), suggesting that periodontitis is not sufficient to induce a functional disorder of the liver of rats in the absence of systemic diseases.

Non-alcoholic fatty liver disease is recognized to be a hepatic manifestation of metabolic disorders (7) and is characterized by hepatocellular steatosis, apoptosis, inflammatory infiltration and fibrosis (8). It has been demonstrated that cholesterol overload (e.g. feeding a high-cholesterol diet) results in not only metabolic disorders but also hepatic fibrosis and inflammation (9). Day & James (10) have suggested a two-hit theory in which the development of non-alcoholic fatty liver disease requires an additional etiological factor as well as steatosis. In their theory, oxidative stress is considered the most likely factor as the 'second hit', a key event for disease progression (10,11). Although periodontal inflammation alone does not induce functional disorder of the liver in rats (6), it may augment liver injury induced by a high-cholesterol diet because systemic oxidative stress is increased by application of a combination of LPS and proteases to the gingival sulcus (12).

The purpose of the present study was to investigate the effects of topical application of a combination of LPS and proteases to the gingival sulcus on hepatic pathological changes and serum parameters of liver function in rats fed a high-cholesterol diet. In addition, since one of the common hepatic cellular responses to periodontal inflammation and cholesterol overload is induction of free radical production (6,13), the changes in hepatic oxidative damage were also examined.

Material and methods

Animals

Twenty-four male Wistar rats (8 weeks old) were used in this study. The animals were housed in steel cages in controlled conditions of 12 h light–12 h dark cycles, 50% humidity and 22–25°C. All experimental procedures were approved by the Animal Research Control Committee of Okayama University Dental School.

Experimental design

Animals were randomly divided into four groups of six rats each. During the experimental period, the first two groups were fed with a normal diet for 8 weeks, and they received topical application of pyrogen-free water (control group) or 25 μg/μL *Escherichia coli* LPS and 2.25 U/μL *Streptomyces griseus* proteases (periodontitis group) to the gingival sulcus for 4 weeks prior to the end of the experimental period (14). The remaining two groups were fed a diet containing 1% cholesterol (w/w) and 0.5% cholic acid (w/w; Oriental Yeast Co., Tokyo, Japan) for 8 weeks, and they received topical application of pyrogen-free water (cholesterol group) or LPS and proteases (combination group) for 4 weeks prior to the end of the experimental period. Lipopolysaccharide (0.5 μL, three doses) and proteases (0.5 μL, three doses) or pyrogen-free water (0.5 μL, six doses) were introduced once a day using a micropipette into the gingival sulcus of both maxillary first molars. The tip of the micropipette was placed close to the gingival sulcus, and 0.5 μL of the solutions (LPS and proteases, or pyrogen-free water) was dropped into the sulcus (14), within 1 h after induction of general anesthesia by inhalation of 2–4% isoflurane delivered in a O_2 gas through a face mask.

Blood collection and measurement of biochemical markers

At the end of the experimental period, blood samples (2 mL) were collected directly from the heart of 24-h-fasted animals under general anesthesia with diethyl ether. Blood was allowed to clot at room temperature for 1 h, and serum was separated by centrifugation at 1500*g* for 15 min. Levels of serum total cholesterol and triglycerides were evaluated with the use of an enzymatic commercial kit (Cholesterol E-test Wako; Wako Pure Chemical Industries, Osaka, Japan; 15). The activities of serum AST and ALT were analyzed with a commercially available assay kit (Wako Pure Chemical Industries). Tumor necrosis factor-α (TNF-α) concentrations in serum were determined with a rat TNF-α enzyme-linked immunosorbent assay (ELISA) kit (Biosource International, Camarillo, CA, USA). Serum levels of C-reactive protein (CRP) were quantified by a highly sensitive ELISA (rat C-reactive protein ELISA test kit; Life Diagnostics, Inc., West Chester, PA, USA).

Measurements of serum oxidative stress

In order to measure serum oxidative stress, the *N*,*N*-diethylparaphenylendiamine reactive oxygen metabolites (d-ROMs) test was performed using the free radical elective evaluator (Diacron, Grosseto, Italy), according to the analysis procedures (16). A 20 μL serum sample and 1 mL of buffered solution (*N*,*N*-diethylparaphenylendiamine, R2 reagent of the

kit, pH 4.8) were gently mixed in a cuvette, and then 10 μL of chromogenic substrate (R1 reagent of the kit) was added to it. After mixing, the cuvette was immediately incubated in the thermostatic block of the analyzer for 5 min at 37°C; 505 nm absorbance was then recorded. The measurement unit was expressed as the Carratelli unit (CARR U). It has been established that 1 CARR U corresponds to 0.08 mg/dL H_2O_2.

Measurement of serum lipid peroxides

Since the formation of hexanoyl-lysine (HEL) in lipid hydroperoxide-modified proteins, including oxidatively modified low-density lipoprotein, was reported as an initial marker for lipid peroxide (17), serum levels of HEL were assessed in triplicate using an HEL ELISA kit (Japan Institute for the Control of Aging, Shizuoka, Japan; 18).

Histological evaluation

The animals were killed at the end of the experimental period, under deep anesthesia with diethyl ether and exsanguination. The maxillary molar regions and liver samples were resected from each rat and immediately fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 1 day. Periodontal tissues were further subjected to decalcification with 10% tetrasodium-EDTA aqueous solution (pH 7.4) for 2 weeks at 4°C. The decalcified periodontal tissue samples and the formalin-fixed liver tissue samples were embedded in paraffin and stained with hematoxylin and eosin.

A single examiner, blind to the treatment assignment, performed the following histometric analyses using a light microscope. Periodontal sections stained with hematoxylin and eosin were used to evaluate the degree of periodontitis. The distances between the cemento-enamel junction (CEJ) and the alveolar bone crest (level of alveolar bone), and between the CEJ and the most apical portion of the junctional epithelium (degree of rete ridge elongation of junctional epithelium) were measured with a microgrid at a magnification of ×200 (6). The number of polymorphonuclear leukocytes (PMNs) and blood vessels in two standard areas (0.05 mm × 0.05 mm each) of the connective tissue subjacent to the junctional epithelium was determined at a magnification of ×400 (6). Three sections were selected from each rat and analyzed.

Liver pathology was scored as described in a previous study (17), as follows: steatosis (the percentage of liver cells containing fat), $<25\% = 1+$, $<50\% = 2+$, $<75\% = 3+$ and $>75\% = 4+$; and inflammation and necrosis, one focus per low-power field $= 1+$, two or more $= 2+$.

Measurements of hepatic levels for 8-hydroxydeoxyguanosine

Mitochondrial DNA was isolated from rat liver with a DNA extraction kit (Wako Pure Chemical Industries). Isolated mitochondrial DNA was analyzed by a competitive ELISA method with the use of an '8-hydroxydeoxyguanosine (8-OHdG) check' kit (Japan Institute for the Control of Aging; 18).

Statistical analysis

Data are presented as means ± SD. Comparisons between the groups of rats (the control vs. the cholesterol, periodontitis or combination groups; and the cholesterol or periodontitis vs. the combination groups) were made by the Mann–Whitney *U*-test. Since five pairwise comparisons were performed, the statistically significant level was set to 0.01 according to Bonferroni's correction. All calculations were performed using a statistical software package (SPSS 15.0J for Windows; SPSS Japan, Tokyo, Japan).

Results

There were no significant differences among the four groups in terms of food consumption and body weight during the experimental period.

Serum levels of total cholesterol, AST, ALT, CRP and HEL in the cholesterol group were significantly higher than those in the control group (Table 1). For the periodontitis group, values of CRP, d-ROMs and HEL were significantly higher than those for the control group. All the serum parameters except triglycerides in the combination group were significantly higher than those in the control group. The level of serum TNF-α in the combination group was higher than those in the cholesterol and periodontitis groups.

Linear distances between the CEJ and the most apical portion of the junctional epithelium and between the

Table 1. Results of serum parameters

	Control group ($n = 6$)	Cholesterol group ($n = 6$)	Periodontitis group ($n = 6$)	Combination group ($n = 6$)
Total cholesterol (mg/dL)	56 ± 9	146 ± 17*	66 ± 10	140 ± 55*‡
Triglycerides (mg/dL)	22 ± 8	18 ± 8	24 ± 10	24 ± 9
Aspartate aminotransferase (i.u./L)	106 ± 22	192 ± 46*	92 ± 20	205 ± 99 *‡
Alanine aminotransferase (i.u./L)	21 ± 4	42 ± 19*	19 ± 3	101 ± 118*‡
Ratio of aspartate aminotransferase to alanine aminotransferase	5.3 ± 1.6	4.9 ± 1.1	4.8 ± 0.8	2.9 ± 1.1*‡
Tumor necrosis factor-α (pg/mL)	6.0 ± 2.3	9.3 ± 3.4	6.0 ± 2.0	18.7 ± 5.4*†‡
C-reactive protein (mg/mL)	0.8 ± 0.3	2.2 ± 0.9*	2.0 ± 0.6*	2.2 ± 0.5*
N,*N*-diethylparaphenylendiamine reactive oxygen metabolites (CARR U)	294 ± 30	443 ± 184	411 ± 45*	697 ± 203*‡
Hexanoyl-lysine (nmol/L)	6.7 ± 0.7	8.6 ± 0.8*	11.1 ± 3.0*	12.9 ± 6.1*

Values are means ± SD.
*$p < 0.01$ compared with the control group, using Mann–Whitney *U*-test.
†$p < 0.01$ compared with the cholesterol group, using Mann–Whitney *U*-test.
‡$p < 0.01$ compared with the periodontitis group, using Mann–Whitney *U*-test.

Table 2. Histological analysis of rat periodontal tissue

	Control group ($n = 6$)	Cholesterol group ($n = 6$)	Periodontitis group ($n = 6$)	Combination group ($n = 6$)
Linear distance between the CEJ and apical portion of the junctional epithelium (μm)	0 ± 0	50 ± 21*	154 ± 26*	182 ± 25*†
Linear distance between the CEJ and alveolar bone crest (μm)	560 ± 61	720 ± 56*	686 ± 108	837 ± 152*
Polymorphonuclear leukocyte density (number per 0.05 mm × 0.05 mm field)	1.4 ± 0.5	1.9 ± 0.3	2.3 ± 0.2*	2.6 ± 0.4*
Blood vessel density (number per 0.05 mm × 0.05 mm field)	1.3 ± 0.3	2.2 ± 0.3*	2.1 ± 0.2*	2.2 ± 0.4*

Values are means ± SD.
*$p < 0.01$ compared with the control group, using Mann–Whitney *U*-test.
†$p < 0.01$ compared with the cholesterol group, using Mann–Whitney *U*-test.

CEJ and alveolar bone crest and blood vessel density in the cholesterol group were larger than those in the control group (Table 2). Linear distance between the CEJ and the most apical portion of the junctional epithelium and densities of PMNs and blood vessels in the periodontitis group were larger than those in the control group. Moreover, all four histological parameters for the combination group were larger than those in the control group.

In the control group, few pathological changes were observed in the liver (Fig. 1A). The cholesterol group showed moderate hepatic steatosis (Fig. 1B). In the periodontitis group, hepatocytes with small fatty droplets and scattered foci of inflammatory cell infiltration were observed (Fig. 1C). The degree of hepatic steatosis and inflammation in the combination group was greater than those in the other groups (Fig. 1D). The cholesterol group showed higher steatosis and inflammation scores for the liver than the control group (Table 3). The inflammation score in the periodontitis group was higher than that in the control group. Furthermore, the steatosis and inflammation scores in the combination group were higher than those in the cholesterol group.

The level of mitochondrial 8-OHdG in the liver was higher in the cholesterol and periodontitis groups than in the control group (Fig. 2). The hepatic level of mitochondrial 8-OHdG in the combination group was higher than those in both the cholesterol and the periodontitis groups.

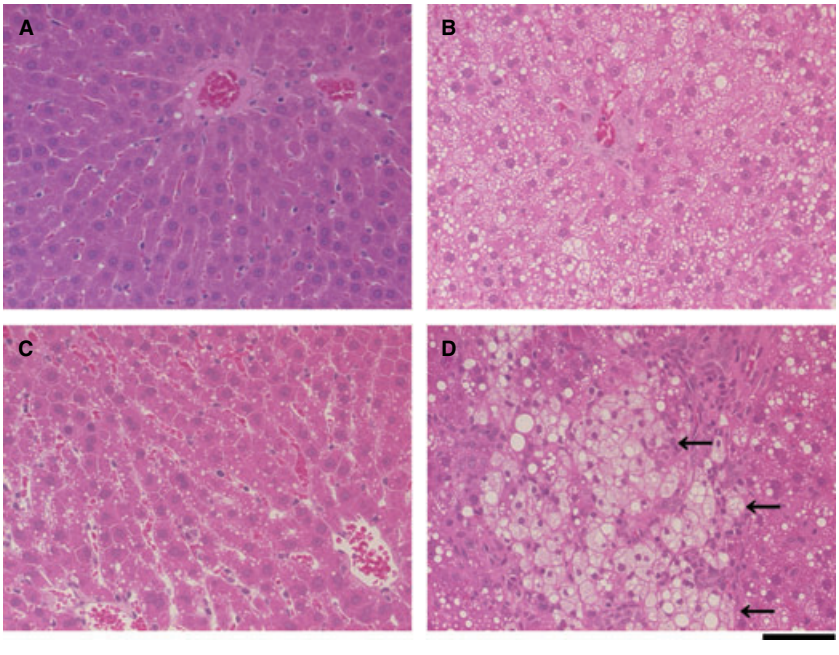

Fig. 1. Liver from rats in the control, cholesterol, periodontitis and combination groups stained with hematoxylin and eosin. Few pathological changes were observed in the control group (A). The periodontitis group showed slight fatty change, characterized by small droplets in the hepatocytes (C). The fatty changes of cholesterol (B) and combination groups (D) were more severe than those of the periodontitis group (C). Focuses of hepatocytes containing fat vacuoles (arrows) are more common in the combination group (D) than any other groups. Scale bar represents 50 μm.

Discussion

Feeding a high-cholesterol diet increased serum levels of AST and ALT and scores of inflammation and steatosis in the liver. In addition, a combination of high-cholesterol diet and topical application of a combination of LPS and proteases reduced the ratio of AST to ALT in serum and increased the 8-OHdG concentration in liver tissue. A decrease in the ratio of AST to ALT in serum and increase in 8-OHdG concentration in liver indicate hepatic impairment (5) and oxidative DNA damage (19), respectively. Since additive effects were evident, topical application of a combination of LPS and proteases might contribute to exacerbate impairment and oxidative damage in the liver.

Topical application of a combination of LPS and proteases to the gingival sulcus increased the steatosis in rats fed a high-cholesterol diet. This result agrees with a proposed theory in which endotoxemia resulting from intestinal bacterial overgrowth contributes to progression of steatosis to

Table 3. Inflammation, steatosis and necrosis scores in rat liver

	Control group ($n = 6$)	Cholesterol group ($n = 6$)	Periodontitis group ($n = 6$)	Combination group ($n = 6$)
Steatosis score				
<25% (1+)	6	0	3	0
25–50% (2+)	0	1	2	0
50–75% (3+)	0	5	1	4
>75% (4+)	0	0	0	2
Inflammation score				
0	6	0	0	0
1+	0	4	6	0
2+	0	2	0	6
Necrosis score				
0	6	3	4	2
1+	0	3	2	3
2+	0	0	0	1

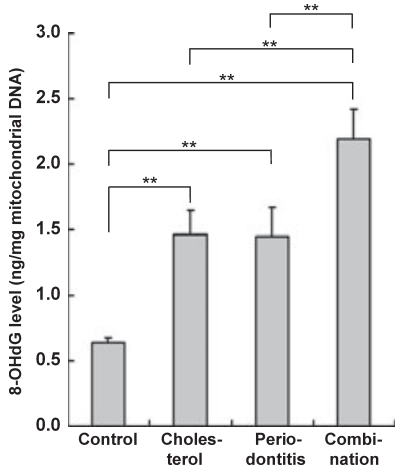

Fig. 2. Hepatic levels of 8-OHdG in the control, cholesterol, periodontitis and combination groups. $^{**}p < 0.001$, pairwise comparison using Mann–Whitney *U*-test.

non-alcoholic steatohepatitis, which represents an advanced stage of fatty liver disease (20). Other studies have indicated that hyperlipidemia frequently accompanies infectious disease (21). The results of the present study suggest that periodontal infection contributes to non-alcoholic fatty liver disease. If the relation is confirmed in human studies in the future, medical colleagues and patients with non-alcoholic fatty liver disease may consider periodontal health as an important factor for control of non-alcoholic fatty liver disease. Moreover, it may be recommended that patients with non-alcoholic fatty liver disease be referred to dentists for checking and treatment of periodontal inflammation.

The exact pathological mechanisms of non-alcoholic fatty liver disease have not been fully elucidated; however, studies suggest that oxidative stress is involved in its pathogenesis. For instance, a placebo-controlled trial involving antioxidants (vitamin E and vitamin C treatment) showed an improvement of fibrosis in patients with non-alcoholic fatty liver disease (22). It is also known that oxidative stress can progress liver injury from steatosis to steatohepatitis mainly by three mechanisms: lipid peroxidation, cytokine induction and Fas ligand induction (23). In the present study, lipid peroxidation and oxidative stress might be responsible for liver injury in the combination group because serum levels of HEL and d-ROMs and liver levels of 8-OHdG were elevated. The results are consistent with studies in which parameters of oxidative stress, such as malonyldialdehyde (24), 4-hydroxynonenal (24) and thioredoxin (25) in blood and 4-hydroxynonenal and 8-OHdG in liver (26), were elevated in patients with non-alcoholic fatty liver disease.

Recent studies demonstrated that a significantly higher level of blood markers of oxidative stress was observed in chronic periodontitis patients than in periodontally healthy subjects (27,28). These findings suggest that periodontal inflammation increases the circulating markers of oxidative stress in chronic periodontitis patients, but it is unclear how such conditions could be detrimental to general health. The present study supports the concept that increased circulating markers of oxidative stress following periodontal inflammation could augment liver injury. In patients with hepatic steatosis, periodontal inflammation may be a contributing factor for progression of non-alcoholic fatty liver disease. In such cases, evaluation of liver enzymes and oxidative stress in the clinic is recommended. Furthermore, the use of anti-oxidants (e.g. vitamin C treatment) in addition to periodontal treatment may be effective in preventing progression of non-alcoholic fatty liver disease in periodontitis patients.

Other mechanisms might also be involved in enhancing steatosis and inflammation in the liver induced by topical application of LPS and proteases. Topically applied LPS might directly affect liver cells through the systemic circulation. An increase in circulating LPS has been reported in our previous study using the same rat model of periodontal inflammation (6). An increased number of blood vessels and the extension of blood vessels were found in the gingiva of the periodontitis and combination groups. These microvascular changes would enable entry of LPS from the gingival connective tissue into the systemic circulation. In fact, LPS applied into the gingival sulcus is transferred to blood vessels 2 h after application (29). Studies have shown that LPS elicits a wide variety of host defense responses to severe tissue injury, including liver injury in many models (30).

Inflammatory cytokines, such as TNF-α, which are produced in periodontal inflammation, might directly affect liver cells. Steatosis is associated with increased TNF-α (20), the level of which was elevated in the serum of rats fed a high-cholesterol diet and with topical application of a combination of LPS and proteases in this study.

Our previous study, using the same rat model and the same study design, showed that high dietary cholesterol could initiate and augment periodontal inflammation (15), and the results were confirmed by the present study. In

another study, a high-cholesterol diet induced oxidative damage in the periodontium (31). Moreover, the present study showed that periodontal inflammation augmented steatosis, inflammation and oxidative damage in the liver. These results suggest that oxidative damage of both periodontium and liver was induced additionally by a high-cholesterol diet and periodontitis. For example, cholesterol overdose induces and/or exacerbates periodontal inflammation, the periodontal inflammation may induce local production of reactive oxygen species and cytokines, and the reactive oxygen species and cytokines may enter the systemic circulation and directly affect the liver.

The degree of liver injury, i.e. scores of inflammation, steatosis and necrosis of the liver, in the periodontitis group was less severe than those observed in the rat model of periodontal disease in our previous study (6). The difference might be ascribed to the period of application of a combination of LPS and proteases to the gingival sulcus: 4 weeks in the present study, compared with 8 weeks in the previous study.

Lipopolysaccharides from *E. coli* and proteases from *S. griseus* were applied to rat gingival sulcus in the present study. These bacterial species are not generally considered to be periodontal pathogens. Although both *E. coli* LPS and LPS from periodontopathic bacteria such as *Porphyromonas gingivalis* have similar effects on the induction of oxidative stress in gingival cells (32), a recent study suggested that *E. coli* LPS, but not *P. gingivalis* LPS induced inflammatory responses in the heart/aorta (33). This is the potential limitation of this study. However, the application of *E. coli* LPS and *S. griseus* protease provide a rat periodontal disease model with high repeatability, and the use of commercial products ensures more uniform experimental conditions than with custom-made products (14,34).

In addition, the present investigation was not a longitudinal study, and changes in parameters were not evaluated over time. Longitudinal studies will be needed to examine the causal relationships among periodontal inflammation, a high-cholesterol diet and hepatic injury.

In conclusion, local application of LPS and proteases to the gingival sulcus augments impairment and oxidative damage of the liver induced by feeding rats with a high-cholesterol diet.

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