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Original Article

An experimental hyperlipidemia model with periodontitis in mice

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Abstract: Purpose: In many animal models and clinical trials, the relationship between periodontitis and hyperlipidemia is bidirectional and interlinked. In this study, an experimental hyperlipidemia model with periodontitis in mice is introduced. Methods: C57BL/6J mice were assigned into group A and B and Apolipoprotein E-deficient ($ApoE^{-/-}$) mice into group C. After 4 weeks of a high fat diet (HFD), group B and C were ligated on the maxillary second molar tooth, and mice were sacrificed after 8 weeks of the HFD. Levels of lipids, interleukin (IL)-6, IL-10, and tumor necrosis factor (TNF)-α in serum after 0, 4, and 8 weeks were determined. Alveolar bone loss (ABL) was assessed under stereomicroscope. Maxillary bones and atherosclerotic lesion area in the aorta were collected for hematoxylin and eosin (HE) staining. Results: After feeding with a HFD for 4 weeks, group C demonstrated dramatic increases in serum lipid levels. The ABL and levels of IL-6, IL-10, and TNF-α in group C was significantly higher than those of group A and B ($P<0.05$). Atherosclerotic lesions were observed in group C. Conclusions: These data demonstrate that an experimental hyperlipidemia model with periodontitis in mice is successfully established by ligation in $ApoE^{-/-}$ mice. This method is economical and time saving, and worthy of more general application.

Keywords: Hyperlipidemia, periodontitis, mouse model

Introduction

Periodontal disease is an inflammatory response caused by infection of the periodontal pocket arising from the accumulation of subgingival plaque. Common signs of periodontal disease include: gingivitis, alveolar bone destruction, tooth mobility, and tooth loss. Periodontal diseases have been considered as possible risk factors for many systemic diseases such as cardiovascular diseases and diabetes mellitus [1]. Hyperlipidemia, a common health problem in adult people especially in the elderly, generates more and more adverse effects on general health. Much emphasis has been placed on the influence of hyperlipidemia on general inflammation and cardiovascular disease, especially atherosclerosis [2].

An association between hyperlipidemia and periodontitis has been noticed in recent years. Epidemiological and clinical studies have suggested that the mean values of pocket depth, clinical attachment level (CAL), plaque index, and bleeding on probing for the hyperlipidemia patients were significantly higher than in those patients that were systemically fit. Total cholesterol and low-density lipoprotein cholesterol levels were significantly and positively associated with CAL [3]. Patients with hyperlipidemia receiving statins had significantly lower gingival index and probing depth compared with those receiving no treatment [4]. Subjects with periodontitis showed significantly increased serum cholesterol and LDL levels compared to subjects with healthy periodontium [5]. Intensive periodontal therapy showed a decrease in total and LDL cholesterol in systemically healthy subjects suffering from severe generalized periodontitis, which suggesting that periodontitis patients are more likely to suffer from hyperlipidemia [6]. In our previous study we demonstrated that hyperlipidemia compromises the homing efficiency of systemically transplanted bone marrow stromal cells (BMSCs) and inhibits mandibular bone regeneration [7]. In the

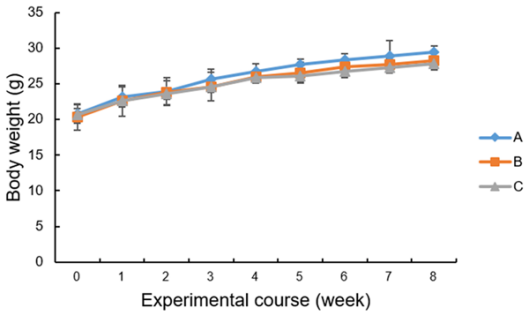

Figure 1. Body weight measurement in experimental animals (n=10). No significant difference in body weight was detected between the three groups throughout the experimental period.

future, we will work to clarify the contribution to tissue regeneration and immunoregulation of MSCs on periodontitis in individuals with hyperlipidemia.

Because of the complexity of clinical trials, establishing reliable animal models is particularly important. A large number of researchers have studied experimental models of periodontitis and hyperlipidemia, respectively. Numerous animal models in different species such as mice, rats, rabbits, hamsters, ferrets, canines and primates have been used for modeling human periodontal diseases and treatments [8-10]. Among these models, the mouse model of ligature-induced periodontitis is one of the most frequently employed animal models to understand the molecular mechanism underlying the onset and progression of periodontitis, as well as to test the efficacy of novel interventions against pathogenic outcomes of periodontitis [11]. In previous studies, rats, pigs, hamsters, and the Mongolian gerbil have been used as models of hyperlipidemia [12-14]. ApoE$^{-/-}$ mice have been a widespread model of severe hyperlipidemia and spontaneous atherogenesis. ApoE$^{-/-}$ mice can acquire hyperlipidemia under a normal diet. Feeding ApoE$^{-/-}$ mice with a HFD leads to a severe shift of serum cholesterol levels [15, 16]. However, research about the establishment and characteristics of this experimental hyperlipidemia model with periodontitis in mice is yet insufficient. In this study, we established an experimental hyperlipidemia model with periodontitis in ApoE$^{-/-}$ mice with an atherogenic high-fat diet and 4-week placement of ligatures on the second maxillary molar tooth, which is considered worthy of generalized use due to lower cost and time required.

Materials and methods

Animals and diet

8-week-old male ApoE$^{-/-}$ mice on C57BL/6J background and C57BL/6J mice with the same gene background (Peking University Health Science Center, Beijing, China) were all fed on a high-fat/high-cholesterol/cholate diet (15%/1.25%/0.5%, respectively). All mice were housed in individual cages under controlled temperatures (24±1.0°C) in a 12 h light/dark cycle and were provided free access to the HFD and aseptic water. C57BL/6J mice were randomly assigned into group A and B and ApoE$^{-/-}$ mice into group C, with 10 mice in each group. The body weight of these animals was recorded every week. Fasting blood samples were taken from the angular vein of 8, 12, 16-week-old animals to determine serum lipid levels including triglyceride (TG), total cholesterol (TC), high density lipoprotein (HDL), and low density lipoprotein (LDL) using an autoanalyzer (Hitachi, Japan). The serum IL-6, IL-10, TNF-α levels were determined by ELISA kit (Elabscience, China) according to manufacturer's instructions. This study was conducted in conformity with the Animal Care and Use Committee of Qingdao University (Qingdao, Shandong Province, China).

Induction of periodontitis

After 4 weeks of HFD, group A was left untreated and the other two groups had their maxillary second molar tooth ligated and were sacrificed after 4 weeks (16-week-old). Briefly, after anesthesia by intraperitoneal injection with 10% chloral hydrate (0.35 ml/100 g) both maxillary second molars of animals in group B and C were treated with a 5-0 silk ligature. The procedure was performed gently to prevent damage to the periodontal tissue under the stereomicroscope's assistance. The ligatures remained in place throughout the experimental period.

Bone loss determination

The isolated left maxillary bones were boiled in water at 15 psi for 10 min. Following deflesching, the maxillary bones were subjected to brushing and bleaching. The maxillary bones

Table 1. Comparison of serum lipid levels (mean ± SD, mmol/L) between Group A, B, and C after the 0, 4, and 8 weeks on a HFD (n=10)

	Group A			Group B			Group C		
	0-week	4-week	8-week	0-week	4-week	8-week	0-week	4-week	8-week
TG	0.82±0.21	0.92±0.22	1.55±0.19	0.82±0.22	0.99±0.24	1.62±0.26	1.18±0.21	1.74±0.35	1.91±0.35
TC	1.99±0.14	3.65±0.52	4.16±0.18	1.98±0.14	3.72±0.29	4.49±0.56	8.67±1.39	30.33±7.71	36.6±4.23
HDL	1.51±0.17	2.4±0.20	3.01±0.28	1.58±0.18	2.52±0.21	3.15±0.51	2.54±0.47	4.89±0.62	5.20±0.24
LDL	0.35±0.07	0.84±0.16	1.00±0.10	0.38±0.06	0.99±0.21	1.23±0.31	2.19±0.31	9.82±3.03	14.2±4.91

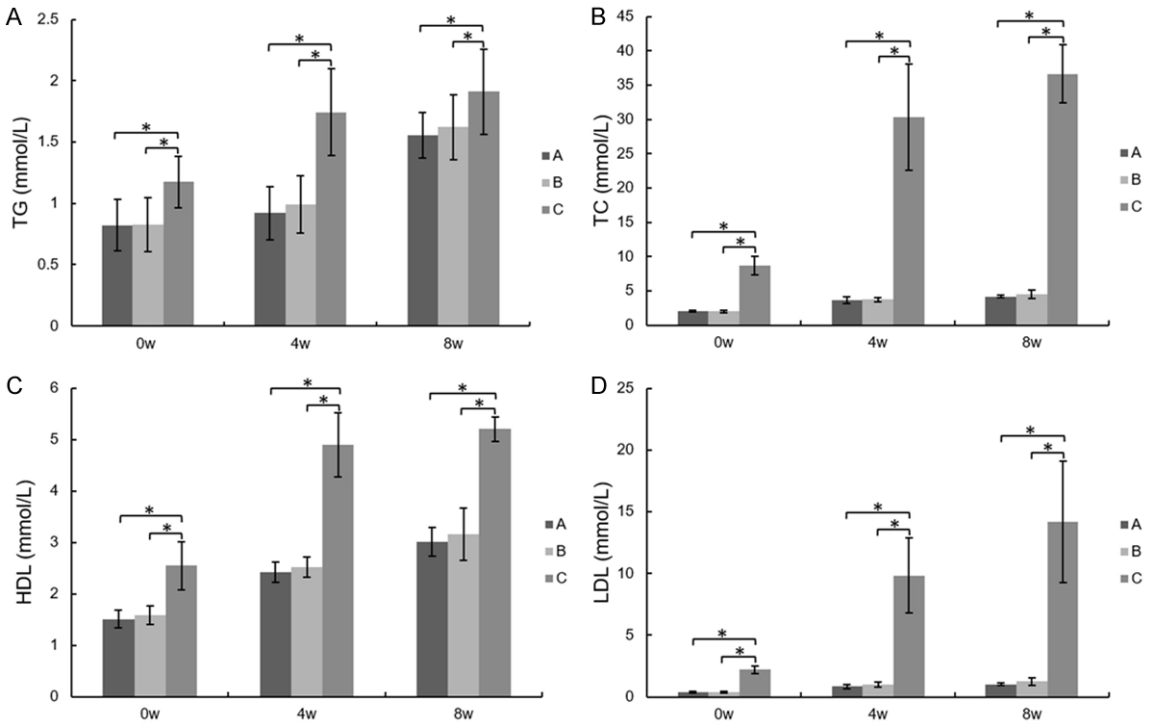

Figure 2. Blood serum lipid levels (n=10). A: Triglyceride (TG). B: Total cholesterol (TC). C: High density lipoprotein (HDL). D: Low density lipoprotein (LDL). *: P<0.05. After fed with the HFD for 4 weeks, group C showed an 8.31-fold increase in TC levels and a 11.69-fold increase in LDL levels was observed when compared with group A. In contrast, only a 2.04-fold increase in serum HDL levels and a 1.89-fold increase in serum TG levels was detected in group C when compared with group A.

were stained with 1% methylene blue. Periodontal bone heights were assessed under a stereomicroscope and bone heights were measured using Image-Pro-Plus 6.0. Measurements were performed on the 6 sites for each of the palatal or buccal side: the first molar (at two sites corresponding to disto-palatal or disto-buccal groove, and distal cusp), second molar (three sites corresponding to mesio-palatal or mesio-buccal cusp, palatal or buccal groove, and disto-palatal or disto-buccal cusp) and the third molar (one site corresponding to palatal or buccal cusp). Periodontal bone heights were measured as the sum of distances from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) on the 12 sites on both the palatal and buccal surfaces.

Tissue preparation and histopathological analysis

Following perfusion fixation with 4% paraformaldehyde, the right maxillary bone and the aorta were isolated. The maxillae samples were decalcified in 10% EDTA for 3 to 4 weeks and embedded in paraffin. Tissue sections, 3 μm in thickness, were cut in a medial-distal direction and the most central area of the second molars was picked out to be stained with Hematoxylin and Eosin (H&E). The aorta samples were

Table 2. Comparison of serum IL-6, IL-10, and TNF-α levels (mean ± SD, pg/mL) between Group A, B, and C after the 0, 4, and 8 weeks on a HFD (n=10)

	Group A			Group B			Group C		
	0-week	4-week	8-week	0-week	4-week	8-week	0-week	4-week	8-week
IL-6	49.76±15.57	60.41±6.96	65.31±10.32	48.46±6.82	60.23±9.30	75.11±8.37	60.7±10.11	70.54±8.60	83.65±7.01
IL-10	75.67±17.13	63.03±7.26	55.45±8.65	75.55±8.22	62.79±7.87	72.29±19.85	97.26±25.26	82.62±17.84	92.43±14.53
TNF-α	47.56±8.79	50.82±12.61	55.33±9.27	47.46±9.44	66.58±14.76	69.33±17.77	52.43±15.14	66.58±13.18	90.85±8.67

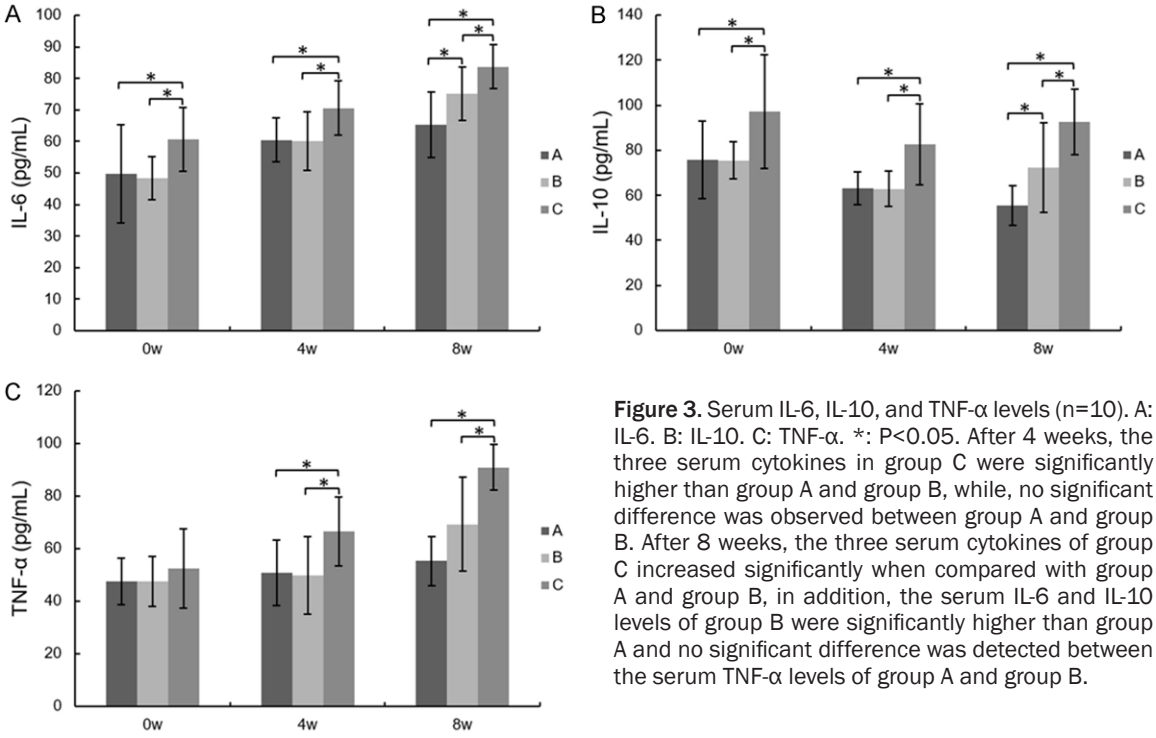

Figure 3. Serum IL-6, IL-10, and TNF-α levels (n=10). A: IL-6. B: IL-10. C: TNF-α. *: P<0.05. After 4 weeks, the three serum cytokines in group C were significantly higher than group A and group B, while, no significant difference was observed between group A and group B. After 8 weeks, the three serum cytokines of group C increased significantly when compared with group A and group B, in addition, the serum IL-6 and IL-10 levels of group B were significantly higher than group A and no significant difference was detected between the serum TNF-α levels of group A and group B.

embedded in paraffin. Tissue sections, 3 μm in thickness, were cut through continuous slicing and stained with H&E.

Statistical analysis

All data were expressed as mean ± SD. Statistical analysis was performed using a statistical package (SPSS 17.0, SPSS Inc, USA). Data were assessed by one-way analysis of variance followed by the Bonferroni correction for multiple comparisons. Values of *p* lower than 0.05 were considered statistically significant.

Results

Body weight

Body weight of both ApoE$^{-/-}$ and C57BL/6J mice increased continuously. No significant difference in body weight was detected between the three groups all through the experimental period (**Figure 1**).

Serum lipid levels

Serum lipid levels in ApoE$^{-/-}$ mice were significantly elevated after fed with HFD for 4 weeks. Briefly, group C showed an 8.31-fold increase in TC levels and an 11.69-fold increase in LDL levels when compared with the group A. In contrast, only a 2.04-fold increase in serum HDL levels and a 1.89-fold increase in serum TG levels were detected in group C when compared with the group A (**Table 1**). These results indicate that a diet-induced hyperlipidemic model was successfully established in ApoE$^{-/-}$ mice after being fed with the HFD for 4 weeks. After 8 weeks of the HFD, serum lipid levels in all groups showed further increases. Furthermore, no significant difference in serum lipid levels

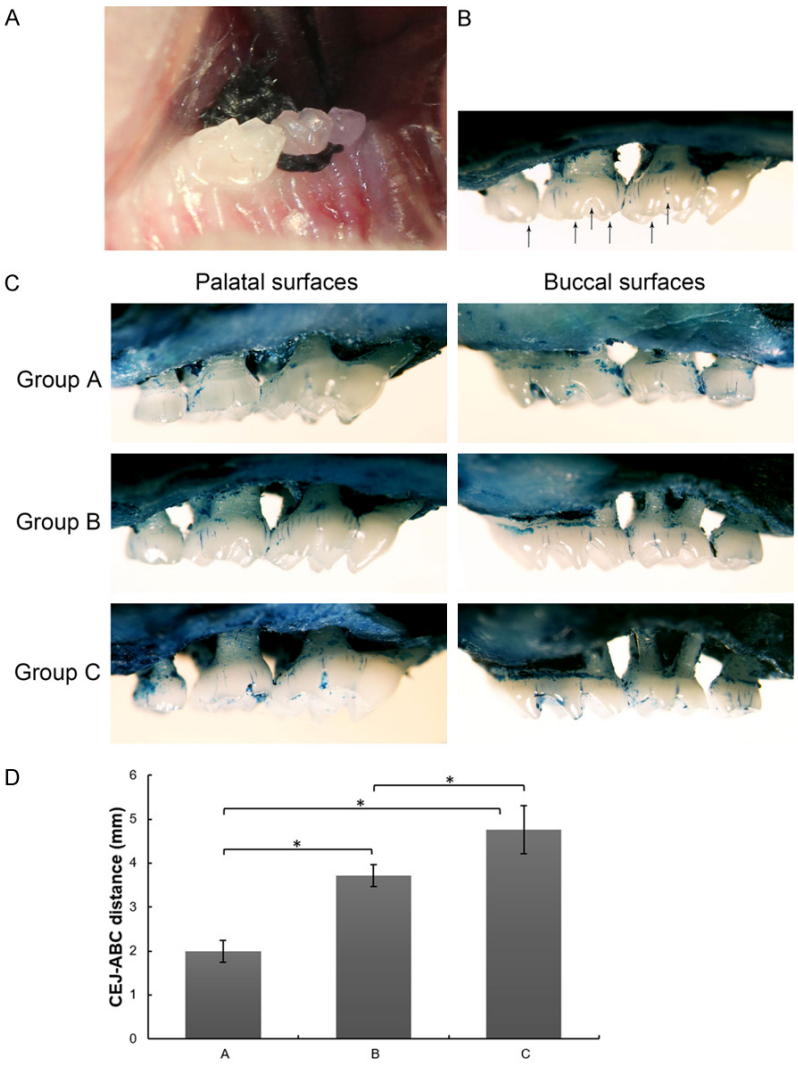

Figure 4. Identification of sites to measure the bone loss. A: To induce a periodontal lesion in mice, a silk ligature was placed around the second molar. B: Measurements were performed on 6 sites total for each of the palatal or buccal side. C: The palatal side and buccal side images of left maxillary bones exhibiting time-dependent bone loss. D: The distances from CEJ to ABC at 12 sites bilaterally was measured and calculated together (n=10). *: $P<0.05$. The alveolar bone resorption level of group B and C were 3.72±0.26 mm and 4.76±0.55 mm respectively, which were significantly higher than group A 1.99±0.25 mm. In the meantime, the alveolar bone resorption level of group C was significantly higher than group B.

was detected between group A and B after the 0, 4, and 8 weeks on a HFD (**Table 1**; **Figure 2**).

Serum IL-6, IL-10, and TNF-α levels

The serum pro-inflammatory cytokines IL-6 and TNF-α levels in three groups increased during the experiment time, while the serum anti-inflammatory cytokines IL-10 levels decreased after 4 weeks. After 4 weeks, the three serum cytokines in group C were significantly higher than group A and group B, at the same time, no significant difference was observed between group A and group B. After 8 weeks, the three serum cytokines of group C increased significantly when compared with group A and group B, in addition, the serum IL-6 and IL-10 levels of group B were significantly higher than group A and no significant difference was detected between serum TNF-α levels of group A and group B (**Table 2**; **Figure 3**).

Alveolar bone loss determination

The distances from CEJ to ABC at 12 sites bilaterally of the isolated left maxillary bones were measured and calculated together, which represented the overall alveolar bone resorption level. The alveolar bone resorption level of group B and C were 3.72±0.26 mm and 4.76±0.55 mm respectively, which were significantly higher than group A 1.99±0.25 mm ($P<0.05$). In the meantime, the alveolar bone resorption level of group C was significantly higher than group B ($P<0.05$) (**Figure 4**).

Histopathological analysis

After H&E staining, the periodontal tissues of group B and C showed typical chronic inflammation, including attachment loss, formation of a periodontal pocket, and alveolar bone resorption. Obvious alveolar bone resorption could be observed in group B and C when compared with group A. Furthermore, alveolar height of group B was higher than group C. In addition, the aorta of group C developed atherosclerotic lesions, while the group A and B showed no abnormalities. ApoE$^{-/-}$ mice had substantial ath-

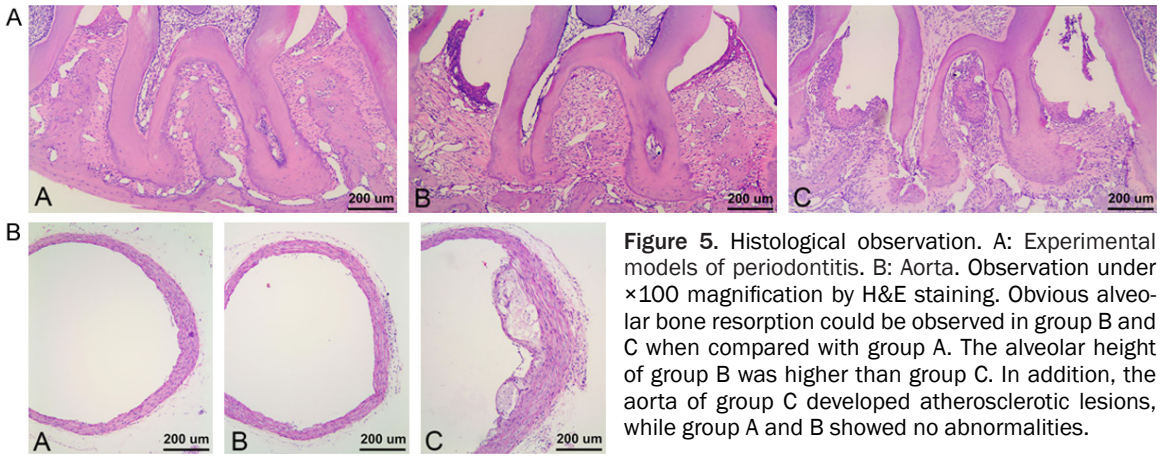

Figure 5. Histological observation. A: Experimental models of periodontitis. B: Aorta. Observation under ×100 magnification by H&E staining. Obvious alveolar bone resorption could be observed in group B and C when compared with group A. The alveolar height of group B was higher than group C. In addition, the aorta of group C developed atherosclerotic lesions, while group A and B showed no abnormalities.

eromas in several locations, H&E staining also demonstrated a proliferative cellular response in the vessel wall (**Figure 5**).

Discussion

In previous studies on the relationship between hyperlipidemia and periodontitis, a high cholesterol diet was always used to establish hyperlipidemia animal model, but the animal's body weight increased at the same time under this diet condition. Normal C57BL/6 mice on high-fat diet gain dramatically more body weight than standard chow diet mice. Obesity interferes with the ability of the immune system to appropriately respond to P. gingivalis infection. Mice with diet-induced obesity (DIO) have a significantly higher level of alveolar bone loss than lean controls after oral infection with P. gingivalis. Furthermore, peritoneal macrophages harvested from mice with DIO exhibit reduced pro-inflammatory cytokine levels compared with lean mice when exposed to P. gingivalis [17, 18]. Therefore, it is necessary to distinguish whether the periodontal response is affected by high fat or obesity in the experiment. In this study, no significant difference in body weight was detected between the ApoE$^{-/-}$ and C57BL/6J mice all through the experimental period, which excluded the impact of obesity on results.

In humans, a defect in the function of ApoE leads to the development of familial type III hyperlipoproteinemia characterized by elevated plasma cholesterol level, the presence of xanthomas, and the development of premature cardiovascular disease [19, 20]. In the 1990s, ApoE$^{-/-}$ mouse models were developed by two independent research groups, and both groups reported that ApoE gene deficiency resulted in severe hyperlipidemia and spontaneous atherosclerotic lesions [15, 21]. In the current study, hyperlipidemia was established using ApoE$^{-/-}$ mice fed with a HFD. Our results showed that after feeding with a HFD for 4 weeks, ApoE$^{-/-}$ mice demonstrated dramatic increases in serum TC and LDL levels when compared with the C57 mice.

The ApoE$^{-/-}$ mouse contains the entire spectrum of lesions observed during atherogenesis and is the first mouse model used to develop lesions similar to atherosclerosis in humans [16]. In this study, after 8-week of HFD, ApoE$^{-/-}$ mice showed obvious atherosclerotic lesions in the aortas. The finding suggests that our model could be used to study atherosclerosis-related diseases.

Elevated levels of lipids and lipid peroxidation can lead to immune system dysfunction, stimulate expression of pro-inflammatory cytokines, cause oxidative stress, delay wound healing, and increase the body's susceptibility to periodontitis [22, 23]. In this study, the ligated ApoE$^{-/-}$ mice showed higher IL-6, TNF-α, and IL-10 levels compared with C57BL/6 mice, which is consistent with previous findings.

The placement of ligatures in effect disrupts periodontal homeostasis by facilitating heavy local accumulation of bacteria. On the other hand, the removal of the ligatures could cause a transition into the resolution phase, and then the animals can be studied with a similar background which can be studied in an appropriate context. For instance, the ligatures could be removed from previously ligated diabetic mice,

to investigate the impact of diabetes on the resolution of periodontal inflammation [11]. After 4-weeks of ligatures, we observed obvious alveolar bone loss in group B and C, which means that periodontitis was established. Most of the previous studies demonstrate the periodontitis animal model using a silk ligature soaked with live Porphyromonas gingivalis suspension, compared which our silk ligation method without bacteria which was more economical [24, 25]. Moreover, the ApoE$^{-/-}$ mice demonstrated more severe periodontal lesions, consistent with previous findings showing that hyperlipidemia could be detrimental to periodontal health [26].

Hyperlipidemia, a well-identified risk factor for cardiovascular disease, is also actively involved in the regulation of bone metabolism shown by most clinical association studies. For example, patients with atherogenic lipid profiles defined as higher TC or LDL levels are considered as having reduced BMD and increased risk of osteopenia compared with those of normal lipid profiles [27, 28]. Furthermore, lipid-lowering agents, which are originally used to reduce serum lipid level and consequently decrease coronary vascular calcification in patients, can also enhance bone mineralization and may reduce osteoporotic fractures [29-31]. When fed with a cholesterol-containing HFD for 12 weeks, ApoE$^{-/-}$ mice displayed reduced cortical bone volume and inhibited bone formation [31]. The relationship between hyperlipidemia and bone metabolism may partly explain how hyperlipidemia affects the progression of periodontal disease. In the present study, group C showed significantly more severe alveolar bone loss than group B.

In conclusion, after a HFD for 4 weeks, the hyperlipidemic mouse model was successfully established using ApoE$^{-/-}$ mice, obvious periodontal damage and aorta atherosclerotic plaques were observed after 4-week placement of ligatures. Our model is a promising small animal model to contribute to better understanding of the bidirectional communication between hyperlipidemia and periodontitis or between atherosclerosis and periodontitis. This model should provide numerous opportunities to study the pathogenesis and therapy of atherosclerosis in a small, genetically defined animal. More importantly, the model is cheaper and time saving, making it more applicable for general usage.

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Disclosure of conflict of interest

None.

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