ORIGINAL ARTICLE

Porphyromonas gingivalis Infection Accelerates Atherosclerosis Mediated by Oxidative Stress and Inflammatory Responses in ApoE-/- Mice

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SUMMARY

Background: The periodontal pathogen *Porphyromonas gingivalis* (*P. gingivalis*) has been proven to accelerate the development of atherosclerosis in apolipoprotein E (ApoE)-deficient mice. In this study, we used an ApoE knock-out (ApoE-/-) mouse model with chronic intravenous infection with *P. gingivalis* to investigate the possible mechanisms of *P. gingivalis*-induced atherosclerosis.
Methods: Eight-week-old ApoE-/- mice were randomly assigned to two groups: (a) ApoE-/- + PBS (n = 8); (b) ApoE-/- + *P. gingivalis* (n = 8). Both of the groups received intravenous injections 3 times per week. After 4 weeks, oxidative stress mediators in serum, heart, aorta, and liver tissues were analyzed by using histology, ELISA, real-time PCR, and Western blot.
Results: Development of atherosclerosis as plaque formation in the aorta has been confirmed upon *P. gingivalis* infection. An abnormal lipid profile was found in the serum [increased amounts of very low-density lipoprotein (vLDL) and oxidized low-density lipoprotein (oxLDL), and decreased amount of HDL] and in some organs including heart, aorta or liver [increased mRNA levels of oxidized low-density lipoprotein receptor-1 (LOX-1) or fatty acid synthase (FAS)]. Meanwhile, aggravated oxidative stress [higher level of reactive oxygen species (ROS) in the serum, and increased mRNA levels of nicotinamide adenine dinucleotide phosphate oxidase (NOX)-2 and/or NOX-4 in the three organs] was observed, as well as enhanced inflammatory responses [increased expression and secretion of C-reactive protein (CRP) in the liver and serum, and increased mRNA levels of cyclooxygenase-2 (COX-2) and/or inducible nitric oxide synthase (iNOS) in the three organs]. Besides, inflammatory mediators including nuclear factor of kappa B (NF-κB) and iNOS showed increased protein levels in the three organs after *P. gingivalis* infection.
Conclusions: These results suggest that chronic intravenous infection with *P. gingivalis* in ApoE-/- mice could accelerate the development of atherosclerosis, possibly associated with mediating oxidative stress as well as inflammatory responses and disturbing the lipid profile.
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KEY WORDS

Porphyromona gingivalis, atherosclerosis, ApoE knockout, lipid profile, oxidative stress, inflammatory responses

LIST OF ABBREVIATIONS

ApoE - apolipoprotein E
COX-2 - cyclooxygenase-2
CRP - C-reactive protein
CVD - cardiovascular disease
FAS - fatty acid synthase
GAPDH - glyceraldehyde 3-phosphate dehydrogenase
HDL - high-density lipoprotein
iNOS - inducible nitric oxide synthase
LDL - low-density lipoprotein
LOX-1 - oxidized low-density lipoprotein receptor-1
LPS - lipopolysaccharide
MI - myocardial infarction
NF-κB - nuclear factor of kappa B
NOX-2 - NADPH (nicotinamide adenine dinucleotide phosphate) oxidase-2
NOX-4 - NADPH (nicotinamide adenine dinucleotide phosphate) oxidase-4
oxLDL - oxidized low-density lipoprotein
PBS - phosphate-buffered saline
ROS - reactive oxygen species
vLDL - very low-density lipoprotein

INTRODUCTION

Periodontal disease is a common disease affecting a great proportion of the population. It is a chronic and destructive disease that affects the tissues surrounding and supporting the teeth and eventually causes tooth loss if untreated. Periodontal disease is associated with Gram-negative anaerobic bacteria in the dental biofilm. Recent epidemiologic studies suggest a link between periodontal infections and systemic diseases including atherosclerosis and other cardiovascular diseases (CVD) [1-6]. Mattila et al. discovered associations between poor dental health and myocardial infarction (MI) in 1989 [5]. Ever since this first evidence, researchers spent more and more efforts to investigate the link between periodontal disease and CVD. Some studies suggest that the degree of coronary atherosclerosis correlates with periodontal disease severity [1,6]. A meta-analysis also indicated a positive association between periodontal disease and CVD, and patients with periodontal disease had a much higher risk of developing CVD [2].

Several Gram-negative obligate anaerobes are involved in gingivitis and periodontitis [7], such as *Campylobacter rectus* [8], *Prevotella melaninogenica* [9], *Tannerella forsythensis* [10], *Prevotella intermedia* [11], *Aggregatibacter actinomycetemcomitans* [12], and *Porphyromonas gingivalis* [13]. Among them, *P. gingivalis* is more thoroughly studied and with confirmed evidence of its access to the systemic circulation and existence in distant organs, as well as with confirmed association with the development of atherosclerosis [14]. Throughout the years, researchers have been focused on the mechanisms of *P. gingivalis*-accelerated atherosclerosis, especially in certain animal models such as apolipoprotein E (ApoE)-deficient mice. These animal models are either infected intravenously by *P. gingivalis* [15] or through the natural oral route [16], and they develop lipid metabolism disorders resulting in vascular and molecular signs of accelerated atherosclerosis development.

Inflammatory responses, oxidative stress, and transcriptional changes are all candidates for the pathological mechanisms of atherosclerosis induced by periodontal diseases. These possible mechanisms associate with each other and work together in the process of atherosclerosis development. Indeed, *P. gingivalis* lipopolysaccharides (LPS) not only induce the up-regulation of the transcriptional nuclear factor κB (NF-κB; an inflammatory mediator), which in turn stimulates the gene expression of inflammatory mediators such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) [17-21], but also up-regulates CD36, one of the scavenger receptors for low-density lipoprotein (LDL) and oxidized LDL (oxLDL), which leads to the accumulation of oxLDL in macrophages [22,23]. Both of these mechanisms can work simultaneously to induce proinflammatory changes in the blood vessels [24,25]. The aim of the present study was to investigate the pathological mechanisms underlying atherosclerosis development in ApoE-knockout (ApoE -/-) mice after chronic intravenous infection with *P. gingivalis*, with respect to the involvement of the reactive oxygen species (ROS) level, the oxidative stress mediators [nicotinamide adenine dinucleotide phosphate oxidase (NOX)-2 and NOX-4], the inflammatory marker [C-reactive protein (CRP)], the inflammatory mediators (NF-κB, iNOS, and COX-2), the lipid profile [high-density lipoprotein (HDL), very low-density lipoprotein (vLDL), and oxLDL] and the lipid metabolism regulatory factors [oxLDL receptor-1 (LOX-1) and fatty acid synthase (FAS)], all of which were reported to be associated with the development of atherosclerosis [26]. The results could have important implications for the management of patients with periodontal diseases.

MATERIALS AND METHODS

Mice

All experiments were approved by the Institutional Animal Care and Use Committee of Peking University Health Science Center (approval number LA201464). Six-week-old male ApoE -/- mice (C57BL/6) were obtained from Vital River Inc., (Beijing, China). The mice were maintained under specific pathogen-free condi-

tions and fed regular chow and sterile water until infection at 8 weeks of age. The animals were divided into two groups: the *P. gingivalis* infection group (n = 8) and the sham control group that received the same injection pattern but only with PBS (n = 8). Both groups received intravenous injections three times per week for 4 weeks starting at 8 weeks of age.

Bacterial cultures and infection
The *P. gingivalis* strain FDC381 was cultured in 5% sheep blood anaerobic basal agar plates (Oxoid Ltd. England) under anaerobic conditions at 37°C for 3 to 5 days until reaching an optical density (OD600) of 1.0, corresponding to 10^9 CFU/mL. Bacterial suspensions were centrifuged at 8,000 g for 20 minutes at 4°C and diluted with PBS (final concentration of 10^8 CFU/100 µL) for intravenous injection (100 µL) three times per week for 4 weeks. The sham control group received PBS only (100 µL/mouse). This dose of bacteria was based on previous studies in rabbits [27,28] and tested in a pilot study, which showed that this dose did not result in mouse death and did induce atherosclerosis (data not shown).

Histological examination with Oil Red O staining
The animals were perfused through the left ventricle with heparinized ice-cold 0.9% PBS for 10 minutes. The heart (including the aortic root) was carefully dissected and embedded in OCT for H&E staining and Oil Red O staining. The other tissue and aortas were snap-frozen in liquid nitrogen and kept at -80°C.
Cryosections of the aortic sinus were prepared for Oil Red O staining, and atherosclerotic lesions were measured using a modified Paigen method [29]. Each section was visualized under an optical microscope (Nikon ECLIPSE-Ci, Japan) and converted to images with a digital camera (Olympus Q Color 5, Japan). Total lesion area and percent of aortic lumen occupied by lesions per section (5-µm thickness) were calculated using the digital Pro-Plus Software 6.0 (Media Cybernetics, USA). Values for 15 sections per animal were averaged and expressed as the percentage of lumen of the proximal aorta occupied by lesions per section per animal. The slides were analyzed in a blinded manner, and the percentage of the aortic lumen occupied by lesions was averaged over 15 sections per animal, expressed as the percentage of the lumen of the proximal aorta occupied by lesions per section, per animal at 12 weeks.

Enzyme-linked immunosorbent assay (ELISA)
After euthanasia, serum samples were isolated from blood obtained by infraorbital puncture by centrifugation at 10,000 rpm for 5 minutes at 4°C. Reactive oxygen species (ROS, TSZ Biosciences, USA), C-reactive protein (CRP, TSZ Biosciences, USA) as well as high-density lipoprotein (HDL, TSZ Biosciences, USA), oxidized low-density lipoprotein (oxLDL, TSZ Biosciences, USA), and very low-density lipoprotein (vLDL, TSZ Biosciences, USA) were measured using comer-

cial ELISA kits.

Western blots
Heart, liver, and aorta protein expressions of NF-κB (p65) and iNOS were determined by western blots using GAPDH as an internal control. Proteins were extracted from frozen tissues with an ice-cold RIPA extraction buffer (w/v = 1/10) containing a protease inhibitor cocktail (Roche). Sample protein concentrations were determined using the Bio-Rad protein assay using BSA as a standard. Supernatant protein (32 µg) was separated with 8% SDS-PAGE and then transferred to a PVDF membrane (300 mA, 90 minutes). Membranes were incubated in 5% BSA-TBST for 1 hour at room temperature and then incubated with primary antibodies against NF-κB (p65) (Abcam, England) or iNOS (Abcam, England) and GAPDH in blocking buffer over night at 4°C. After three washes (3 x 10 minutes) in TBST under gentle agitation, membranes were incubated for 40 minutes with horseradish peroxidase-labeled antibody (Jackson Lab, USA). After further washes, blots were revealed by enhanced chemiluminescence detection reagents (Jackson Lab, USA). The films were scanned and densitometric analyses using Gel Image system (ver. 4.00, Tanon, China). The results were expressed as the ratio between NF-κB or iNOS and GAPDH.

Quantitative real-time PCR
Total RNA was purified from heart, aorta, and liver tissues using an RNeasy Fibrous Tissue Kit (Qiagen, Germany) and an RNeasy Plus Mini Kit (Qiagen). After reverse-transcribing with a Primescript RT Master Mix Kit (Takara Bio, Japan) to generate cDNA, quantitative real-time PCR analysis was performed using the Applied Biosystems 7500 Fast Real-time PCR System (Life Technologies, USA), in accordance with the manufacturer's protocol. Briefly, the reactions contained 10 µL of 2 x SYBR Green (Takara Bio, Japan), 100 nM of each primer, and 30 ng of reverse-transcribed RNA. PCR conditions were: 95°C for 30 seconds, followed by 40 cycles at 95°C for 5 seconds, and 60°C for 34 seconds. After that, dissociation curve analysis was performed to confirm specificity. Each gene was tested in triplicate, and the target RNA was normalized to GAPDH mRNA. Primers are presented in Table 1.

Statistical analysis
The data were presented as mean ± standard error. Unpaired *t*-test was used to compare the two groups. P-values ≤ 0.05 were considered significant. All analysis was performed using SPSS version 11.5 (SPSS Inc., Chicago, IL, USA).

RESULTS

Histological changes in aortic sinus after infection with *P. gingivalis*
To confirm the histological changes in the aortic sinus of this pathological model, both H&E and Oil Red O staining were carried out on the samples from the two groups. H&E staining of the aortic sinus showed that the animals infected with *P. gingivalis* had severely increased atherosclerotic cholesterol, characterized by thickened intima and lipid deposition under the endothelium (Figure 1A). When stained with Oil Red O method, lipid depositions in the aortic sinus was stained red and considered as atherosclerotic lesion development. As shown in Figure 1B, the mice infected with *P. gingivalis* developed much more lipid deposition in the aortic sinus area compared to the control group (almost twice in percentage of the total area; n = 8, $p < 0.01$, *t*-test).

ELISA assay of serum samples indicated abnormal lipid profile together with oxidative stress and inflammation after *P. gingivalis* infection
The oxidative stress level, inflammatory marker, and the lipid profile in the serum from the mice of both groups were tested. ROS, CRP, HDL, oxLDL, and vLDL were measured by ELISA. After 4 weeks of *P. gingivalis* infection, the serum of the ApoE-/- mice showed significantly increased ROS compared to the PBS control group (45.87 ± 3.91 vs. 43.28 ± 3.15 IU/mL, n = 8, $p < 0.05$, *t*-test) (Figure 2A), significantly increased CRP (615.56 ± 57.66 vs. 451.19 ± 22.02 μg/L, n = 8, $p < 0.01$, *t*-test) (Figure 2B), significantly decreased HDL (57.62 ± 3.88 vs. 71.85 ± 14.82 μmol/mL, n = 8, $p < 0.05$, *t*-test) (Figure 2C), significantly increased oxLDL (7.55 ± 1.02 vs. 5.25 ± 0.43 μmol/mL, n = 8, $p < 0.01$, *t*-test) (Figure 2D), and significantly increased vLDL (32.42 ± 5.20 vs. 25.38 ± 7.60 μg/μL, n = 8, $p < 0.05$, *t*-test) (Figure 2E), suggesting that the animals infected with *P. gingivalis* had a redistribution of their lipid profile, showing increased inflammatory responses and oxidative stress.

Increased oxidative stress mediators and inflammatory mediators were detected in heart, aorta, and liver tissues of the animals infected with *P. gingivalis*
The RNA levels of some oxidative stress mediators, inflammatory mediators, and lipid metabolism regulatory factors were observed by real-time PCR in the heart, aorta, and liver tissues from animals of both groups. Each target mRNA was normalized to GAPDH. In the hearts from the mice infected with *P. gingivalis*, there were significant increases in the RNA levels of NOX-2 (3.11 ± 1.21 vs. 1.65 ± 0.71, n = 8, $p < 0.05$, *t*-test), NOX-4 (4.22 ± 1.70 vs. 1.66 ± 0.84, n = 8, $p < 0.01$, *t*-test), iNOS (4.95 ± 2.01 vs. 1.12 ± 0.40, n = 8, $p < 0.001$, *t*-test), COX-2 (1.64 ± 0.65 vs. 1.00 ± 0.32, n = 8, $p < 0.05$, *t*-test), and LOX-1 (2.65 ± 0.81 vs. 1.75 ± 0.72, n = 8, $p < 0.05$, *t*-test), as compared to the PBS

control group (Figure 3A).
In the aorta, the RNA levels of NOX-4 (1.03 ± 0.55 vs. 0.50 ± 0.31, n = 8, $p < 0.05$, *t*-test), iNOS (0.88 ± 0.29 vs. 0.52 ± 0.31, n = 8, $p < 0.05$, *t*-test), COX-2 (1.31 ± 0.60 vs. 0.66 ± 0.34, n = 8, $p < 0.05$, *t*-test), and LOX-1 (1.48 ± 0.75 vs. 0.58 ± 0.23, n = 8, $p < 0.01$, *t*-test) were significantly higher in the infected mice, as compared with the control group; while the NOX-2 RNA levels showed a tendency toward an increase (1.02 ± 0.46 vs. 0.76 ± 0.40, n = 8, $p > 0.05$, *t*-test) (Figure 3B).
The mediators in the liver showed similar results: NOX-2 (0.98 ± 0.15 vs. 0.64 ± 0.20, n = 8, $p < 0.01$, *t*-test), iNOS (0.61 ± 0.26 vs. 1.06 ± 0.42, n = 8, $p < 0.05$, *t*-test), CRP (1.08 ± 0.25 vs. 0.62 ± 0.25, n = 8, $p < 0.01$, *t*-test), and FAS (1.04 ± 0.09 vs. 0.75 ± 0.26, n = 8, $p < 0.05$, *t*-test) were significantly higher in the infected mice compared to the control group, except that NOX-4 only showed a tendency to increase (0.73 ± 0.13 vs. 0.57 ± 0.21, n = 8, $p > 0.05$, *t*-test) (Figure 3C).
Taken together, these results indicated that *P. gingivalis* infection leads to oxidative stress and inflammatory responses throughout the body, especially in the heart, aorta, and liver, causing abnormalities in lipid metabolism.

NF-κB/iNOS signaling might contribute to *P. gingivalis*-induced atherosclerosis
To further investigate the inflammation-related mechanisms of atherosclerotic development caused by *P. gingivalis* infection, western blots for NF-κB and iNOS protein levels were performed in the heart, liver, and aorta from animals of both groups. In the heart, the protein levels of NF-κB were significantly higher in the animals infected with *P. gingivalis* compared to the control group (0.34 ± 0.05 vs. 0.28 ± 0.06 after normalization to GAPDH protein levels, n = 8, $p < 0.05$, *t*-test, Figure 4A), while the protein levels of iNOS also showed a significant increase compared to the control group (0.48 ± 0.13 vs. 0.31 ± 0.13 after normalization to GAPDH protein levels, n = 8, $p < 0.05$, *t*-test, Figure 4A). In the aorta, the protein levels of NF-κB showed significant elevation when compared to the control group (0.43 ± 0.12 vs. 0.32 ± 0.06 after normalization to GAPDH protein levels, n = 8, $p < 0.05$, *t*-test, Figure 4B), and the protein levels of iNOS were also significantly increased in the infected group (0.35 ± 0.04 vs. 0.18 ± 0.06 after normalization to GAPDH protein levels, n = 8, $p < 0.001$, *t*-test, Figure 4B). In the liver, the animals infected with *P. gingivalis* also showed significantly increased protein levels of NF-κB (0.45 ± 0.10 vs. 0.30 ± 0.05 after normalization to GAPDH protein levels, n = 8, $p < 0.01$, *t*-test, as shown in Figure 4C) and iNOS (0.50 ± 0.08 vs. 0.38 ± 0.13 after normalization to GAPDH protein levels, n = 8, $p < 0.05$, *t*-test, Figure 4C) compared to the control group. These results suggest that NF-κB signaling (including the downstream target such as iNOS) was involved in the devel-

Table 1. Primers used for quantitative real-time PCR.

| Primer	Forward	Reverse
LOX-1	TGAAGCCTGCGAATGACGAG	GTCACTGACAACACCCAGGCAGAG
COX-2	TGCCAGGCTGAACTTCGAAAC	GCTCACGAGGCCACTGATACCTA
CRP	GAACTGGCGGGCACTGAACTA	GGAGGTGCTTCAGGGTTCACA
FAS	AGCACTGCCTTCGGTTCAGTC	AAGAGCTGTGGAGGCCACTTG
iNOS	TCGAGCCCTGGAAGACCCACATCT	GTTGTTCTTCTTCCAAGGTGTTTGCCTTAT
NOX-2	GCCCAAAGGTGTCCAAGC	TCCCCAACGATGCGGATAT
NOX-4	ACCCTGTTGGATGACTGGAA	ACCAACGGAAAGGACTGGATA
GAPDH	TGTGTCCGTCGTGGATCTGA	TTGCTGTTGAAGTCGCAGGAG
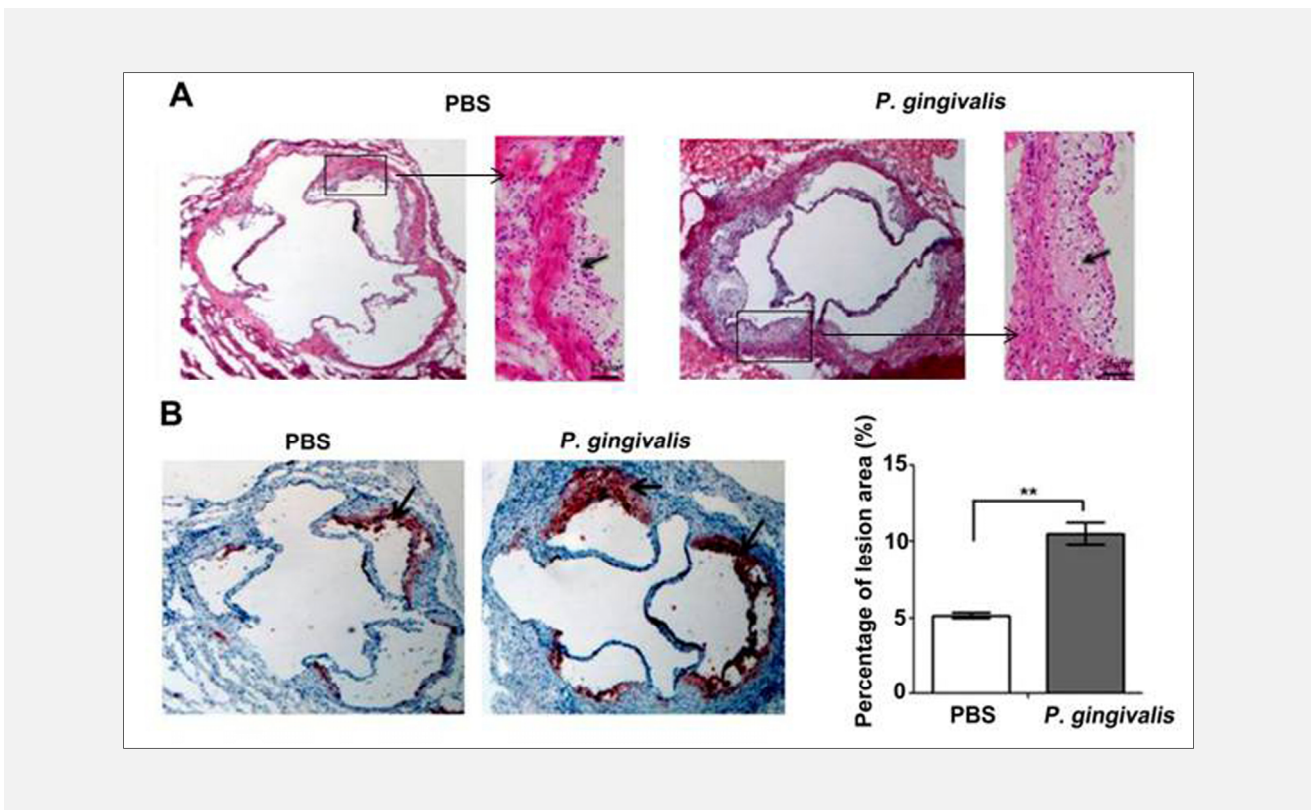
Figure 1. Effects of chronic intravenous infection with *P. gingivalis* on aortic atherosclerosis in ApoE-/- mice.

(A) Representative aortic sinus cross-sections after H&E staining. Scale bar: 25 μm. (B) Representative aortic sinus cross-sections after Oil Red O staining. Aortic atherosclerotic development was reflected by the percentage of lesion areas in aortic sinus. Results were expressed as mean ± standard error, n = 8 in each group, ** - p < 0.01, *t*-test.

opment of atherosclerosis in ApoE-/- mice caused by *P. gingivalis* infection.

DISCUSSION

The present study was designed to investigate the changes in oxidative stress and inflammatory responses during atherosclerotic development in ApoE-/- mice infected with *P. gingivalis*, as the possible underlying
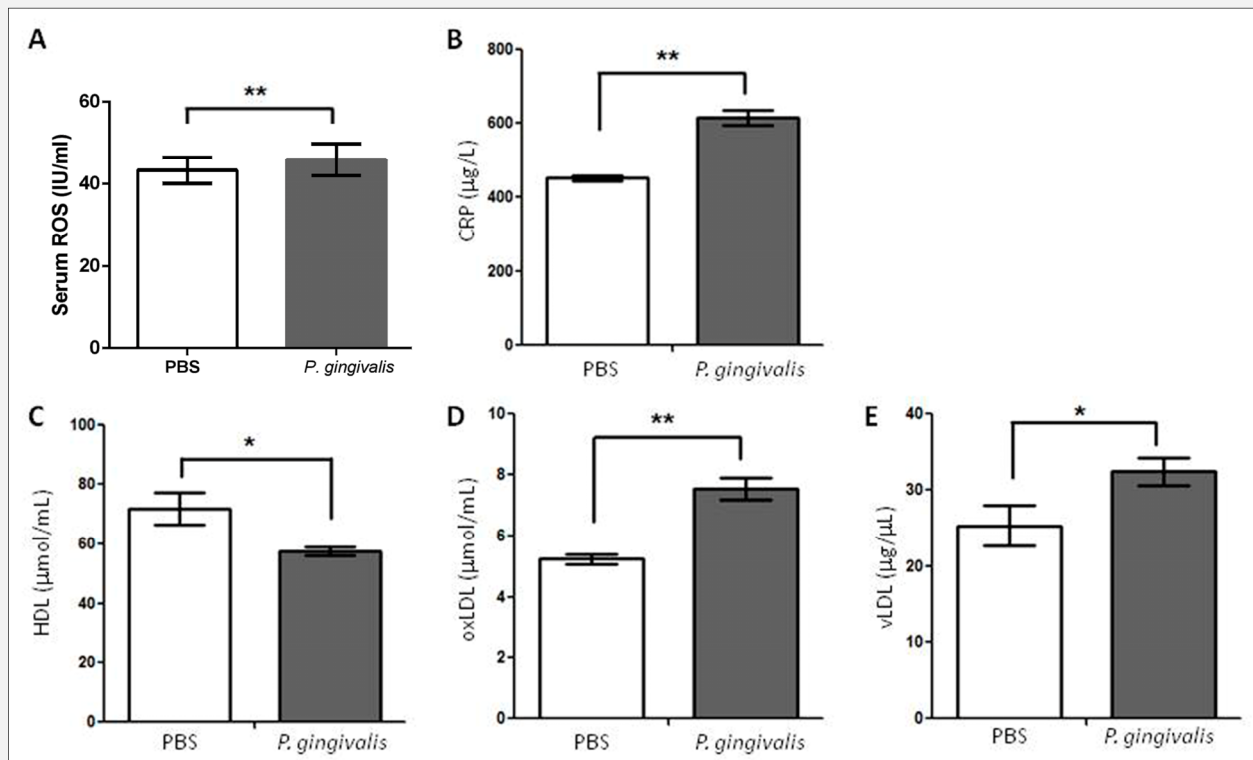
Figure 2. Comparison of oxidative stress, inflammation, and lipid profile (A – ROS, B – CRP, C – HDL, D – oxLDL, E - vLDL) in the serum between the control group and the infected group, as detected by ELISA.

Results were expressed as mean ± standard error, n = 8 in each group, * - p < 0.05, ** - p < 0.01, *t*-test.

mechanisms. The results confirmed that chronic intravenous infection with *P. gingivalis* significantly accelerated atherosclerosis development in the aortic sinus and revealed significant changes in the lipid profile and dramatic increases in oxidative stress in the serum as well as in the liver, heart, and aorta. In addition, there was activation of the NF-κB signaling (significant increase of its targets iNOS and COX-2) in the liver, heart, and aorta. The inflammatory marker CRP was up-regulated in the liver and showed a higher level in the serum. Taken together, the results suggested a possible contribution of the oxidative stress and inflammatory responses to the development of atherosclerosis upon *P. gingivalis* infection.

Among the pathogens responsible for periodontal diseases, *P. gingivalis* is the most common pathogen. It is known to be involved in accelerating atherosclerosis formation [15,16,30] and increasing systemic inflammatory markers [16,31-33]. According to previous studies, *P. gingivalis*, especially its LPS, can promote atherogenesis by changing the serum lipid distribution to a proatherogenic lipid profile. Indeed, LDL, vLDL, and

oxLDL could be induced to cause vascular inflammation and lipid accumulation in macrophages [14]. As shown in the present study, chronic intravenous infection with *P. gingivalis* in ApoE -/- mice significantly changed the serum lipid profile with a significant decrease in HDL, a significant increase in vLDL, and a significant increase in oxLDL compared to the control group. On one hand, *P. gingivalis*-derived LPS can form assemblies with LDL and increase LDL uptake by macrophages [34,35], leading to oxLDL accumulation in macrophages by up-regulating the oxLDL receptor [22,23]. On the other hand, LPS down-regulates lipid catabolism by suppressing liver X receptors in macrophages [36]. With this disturbance of the lipid metabolism and distribution, the macrophages will eventually transform into foam cells. These morphological changes in the blood vessel and lesion development were clearly reflected by the H&E and Oil Red O staining in the present study.

Besides the changes in lipid profile, we believe that oxidative stress plays a major role in the development of atherosclerosis in this animal model of *P. gingivalis* in-
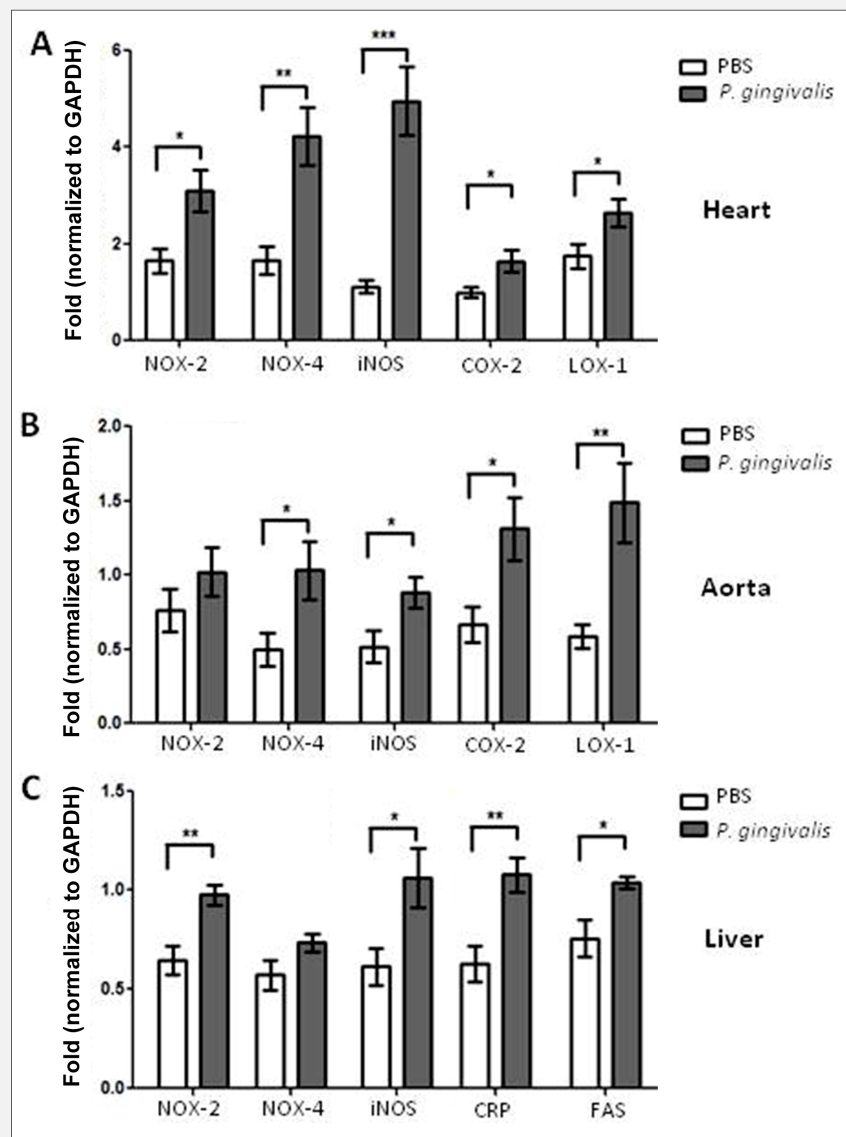
Figure 3. Comparison of the mediators of oxidative stress, inflammation, and lipid metabolism in the heart (A), aorta (B), and liver (C) between the control and infected groups, with regards to relative gene expression levels.

The relative quantity of target mRNA was normalized to the GAPDH mRNA. The mediators detected included: NOX-2, NOX-4, iNOS, COX-2, and LOX-1 in the heart and aorta; NOX-2, NOX-4, iNOS, CRP, and FAS in the liver. Results were expressed as mean ± standard error, n = 8 in each group, * - p < 0.05, ** - p < 0.01, *** - p < 0.001, *t*-test.

fection. Oxidative stress happens when the production of ROS exceeds their clearance by antioxidant defenses. It can cause damage to macromolecules and cells, and plays an important role in the development of atherosclerosis. ROS caused by periodontal pathogens can enhance the oxidative modifications of LDL [37-40], which also helps increase the cholesterol uptake by macrophages and eventually leads to the formation of foam cells [41]. Besides oxLDL, there were dramatic increases of other oxidative stress mediators, including NOX-2 and NOX-4 in the heart, aorta, and liver, suggesting that the oxidative stress caused by *P. gingivalis* infection was a systemic reaction that affected multiple organs.

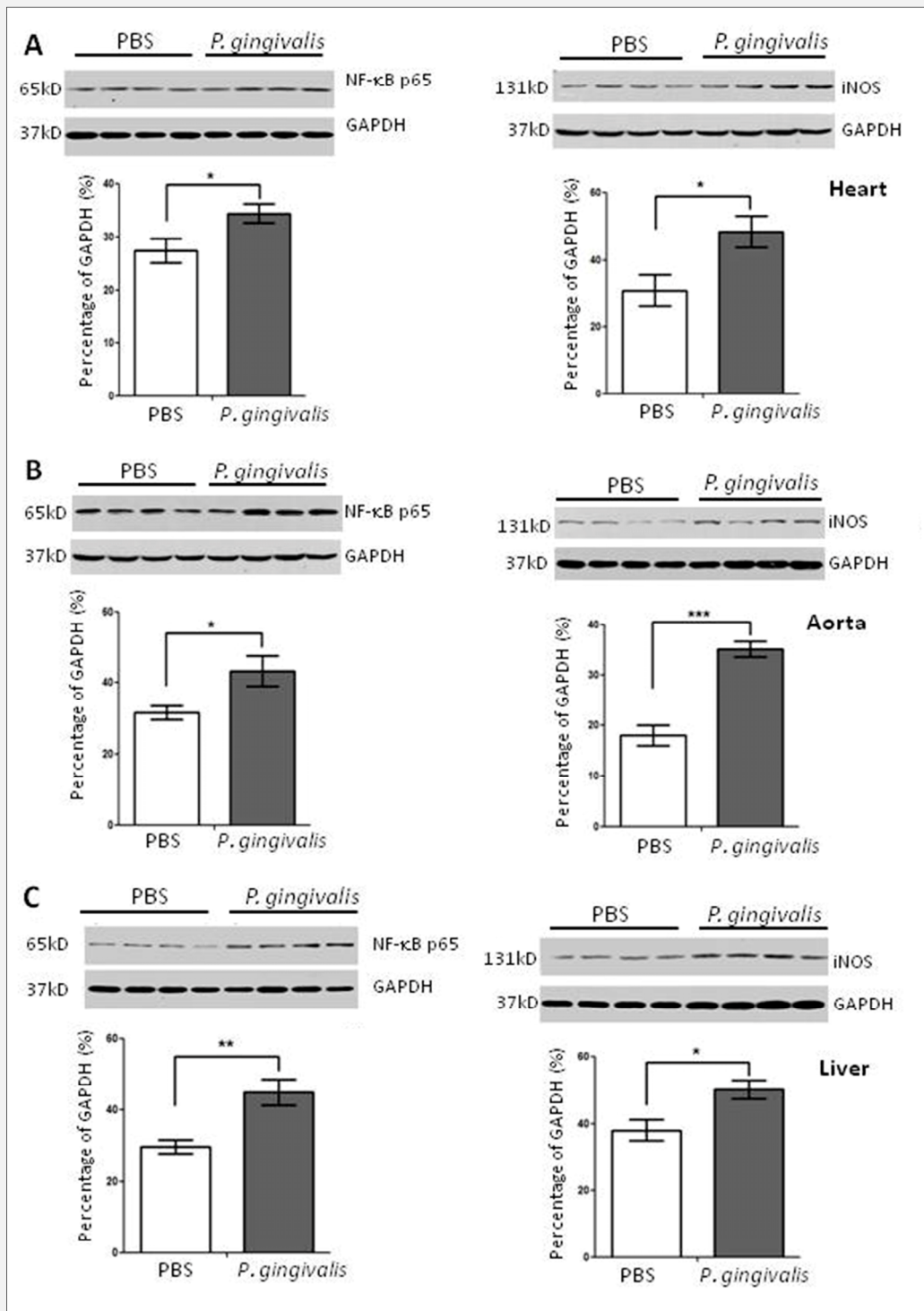On the other hand, western blotting showed that the up-regulation of NF-κB signaling was possibly triggered by

Figure 4. Comparison of the protein levels of NF-κB and iNOS in the heart, aorta, and liver between the control and infected groups.

The relative quantity of protein was normalized to the GAPDH protein. (A) Representative western blot of NF-κB and iNOS in the heart and comparison of the protein levels with GAPDH as the internal control. (B) Representative western blot of NF-κB and iNOS in the aorta and comparison of the protein levels with GAPDH as the internal control. (C) Representative western blot of NF-κB and iNOS in the liver and comparison of the protein levels with GAPDH as the internal control. Results were expressed as mean ± standard error; n = 8 in each group, * - p < 0.05, ** - p < 0.01, *** - p < 0.001, *t*-test.

P. gingivalis as well as the up-regulation of iNOS was possibly triggered by NF-κB in the heart, aorta, and liver. Some studies suggested that NF-κB could also regulate some enzymes that promote the production of ROS, such as NOX-2 [17], iNOS [42-45], and COX-2 [46, 47]. Under physiological conditions, NF-κB exists as an inactive complex with inhibitory proteins (IκB) and stays in the cytosol [48]. When the triggers that can activate NF-κB enter the cell, IκB is phosphorylated, ubiquitinated, and degraded [49-51], which will release NF-κB to enter the nucleus to regulate the expression of a number of target genes. As one of the transcription factors whose binding sites are located in the promoter region of the iNOS gene [19,52-55], NF-κB has the potential of regulating the expression of iNOS and other inducible genes, such as COX-2 [21]. LPS derived by *P. gingivalis* is a well-known exogenous inducer that can stimulate the activation of NF-κB, accelerate the transfer of NF-κB from the cytosol to the nucleus, and therefore induce the gene expression of iNOS [18-20]. Normally, iNOS expressed by cells provides protection against pathogens [56], but the expression of iNOS in macrophages can lead to cell death and may contribute to the development of inflammatory diseases including atherosclerosis [57]. Based on the present study, the pathway involving NF-κB and iNOS might play a major role in the process of atherosclerosis accelerated by *P. gingivalis*. Some studies suggested that oxidative stress markers might also work as cellular messengers and affect cell signaling, including NF-κB [58-60]. So, in this case, NF-κB and oxidative stress mediators might work as each other's enhancer and accelerate the development of atherosclerosis. The detailed interaction between NF-κB signaling and oxidative stress mediators in ApoE-/- mice infected with *P. gingivalis* will be the focus of our future studies.

Based on previous studies as well as the present one, it can be surmised that pathogen infection could induce an "oxidative burst", upon which the oxidative stress and inflammatory responses will promote each other [40]. The aggravated oxidative stress and inflammation will increase lipoprotein oxidation, which closely associated with the development of atherosclerosis [40]. Our results show that *P. gingivalis* infection will evoke oxidative stress and inflammatory responses, which might have impacts on lipid metabolism, ultimately leading to atherosclerosis. Nevertheless, a vast number of factors are involved in the response to oxidative stress and inflammation, and additional studies with a more comprehensive panel of markers and factors are necessary to improve our understanding of *P. gingivalis*-induced atherosclerosis.

The present study suggests that the best way to prevent *P. gingivalis*-induced atherosclerosis would be to prevent the infection itself by promoting periodontal health or by prophylaxis. If this is impossible or if it fails, then interventions aiming to decrease oxidative stress, inflammation, and lipid abnormalities could be a good way to achieve this. Due to their lipid-lowering and pleiotropic effects, statins could be a drug of choice in this context [61-63], but additional studies are necessary to assess their efficacy in the context of *P. gingivalis* infection.

CONCLUSION

In summary, the present work showed that chronic infection with *P. gingivalis* would accelerate the development of atherosclerosis in ApoE-/- mice, possibly associated with mediating oxidative stress as well as inflammatory responses and disturbing the lipid profile. These results provide a potential mechanism of periodontal disease-induced atherosclerosis and a possible pathway to interfere/prevent further development of the disease.

Authors' Contributions:
Y.X. and Q.X.L. designed and conducted the study. Y. X and Y. C provided essential reagents and materials. Y.X., Q.S., and G.J.L. analyzed the data. Y.X., Y.C., and Q.X.L. wrote the paper. Y.X. and Q.X.L. had the primary responsibility for the final content and contributed equally to the study. All authors read and approved the final manuscript. The authors thank Dr. Zhi-Bin Chen for her kind assistance.

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Declaration of Interest:
Authors declared no conflict of interests.

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