Effects of Periodontal Intervention on Levels of Serum High-sensitivity C-reactive Protein and Interleukin 6, and on Carotid Artery in Rats with Chronic Periodontitis and Hyperlipidemia

Miao Miao WANG[1*], Yong ZHAO[1*], Chong WANG[1], Xue Xue SHI[1], Jin Hua GAO[1], Xiu Yun REN[1]

Objective: *To investigate the effects of nonsurgical periodontal therapy on the serum interleukin 6 (IL-6) and high-sensitivity C-reactive protein (hsCRP) and on the carotid artery in rats with chronic periodontitis (CP) and with or without hyperlipidemia (HL).*

Methods: *A total of 29 Sprague-Dawley rats were randomly divided into two groups: group A (CP, n = 14) and B (CP + HL, n = 15), and subjected to the corresponding treatment. Groups A and B were further divided into groups A1/B1 (without periodontal interventions) and A2/B2 (with periodontal interventions). The serum IL-6 and hsCRP levels were evaluated before periodontal intervention and at 1, 3, 5, and 7 weeks after periodontal intervention. The rats were euthanised 8 weeks after the periodontal intervention and the histopathologic changes in the carotid artery were observed.*

Results: *The serum hsCRP and serum IL-6 levels in groups A1 and B1 were elevated with time; they were significantly higher in group B1 than in group A1 ($P < 0.001$) at all time points. The hsCRP and IL-6 levels in groups A2 and B2 increased with time and reached the maximum level 1 week after the second intervention, and then gradually decreased. Atherosclerotic plaques, fibrous cap, and calcium salt deposits were apparent in the rats of group B1, whereas no obvious atherosclerotic changes were observed in the rats of groups A2 and B2.*

Conclusion: *Periodontal interventions resulted in acute, short-term systemic inflammation. However, it was beneficial in long-term as it improved the carotid artery integrity.*

Key words: *atherosclerosis; high-sensitivity C-reactive protein; interleukin 6; periodontal intervention; periodontitis*

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Periodontitis, a common chronic multifactorial infectious disease, is characterised by the presence of bacterial plaque and its byproducts in a susceptible host. It is one of the major causes of tooth loss, especially in elderly individuals. Studies have reported the relationship between periodontitis and several chronic inflammatory diseases such as diabetes mellitus, rheumatoid arthritis, and cardiovascular disease (CVD)[1]. It has been shown that patients with periodontitis have increased risk (1.24 to 1.34 times) of developing CVD[2]. Furthermore, there might be additive effects as both periodontitis and CVD are chronic inflammatory diseases[3].

The C-reactive protein (CRP), an inflammatory biomarker, is predominantly synthesised in hepatocytes as an acute-phase reactant[4]. It has also been used as a marker to predict primary and secondary adverse cardiovascular events[5]. The cumulative exposure to high-sensitivity C-reactive protein (hsCRP) is dose dependently associated with a subsequent increased risk

1 Department of Periodontology, Shanxi Medical University School and Hospital of Stomatology, Taiyuan, Shanxi Province, P.R. China.
* These two authors contributed equally to this work.

Corresponding author: Dr Xiu Yun REN, Department of Periodontology, Shanxi Medical University School and Hospital of Stomatology, 63# Xinjian South Road, Taiyuan 030001, Shanxi Province, P.R. China. Tel: 86 13835133832; Fax: 86 351 4690838. Email: rxy611@163.com.

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of CVD and myocardial infarction[6]. A high serum CRP level has also been observed in patients with chronic periodontitis (CP) or aggressive periodontitis[7,8].

Interleukin-6 (IL-6) is an important proinflammatory cytokine involved in the regulation of host response to tissue injury and infection. It is produced by various cell types such as monocytes, fibroblasts, osteoblasts and vascular endothelial cells in response to inflammatory challenges[9]. Elevated plasma levels of IL-6 are associated with unstable angina and CVD, as well as to other cardiovascular risk factors[10]. Epidemiological studies have suggested the association between periodontal diseases and CVD. A recent systematic review on the association between periodontitis and atherosclerotic CVD provided moderate evidence that periodontal treatment reduces systemic inflammation; as evidenced by the reduction of CRP and improvement in both clinical and surrogate measures of endothelial function[11]. However, there is still no direct evidence to prove that periodontal treatment can benefit CVD. There are ethical limitations to generate this type of evidence given that one cannot obtain cardiovascular specimens after periodontal treatment.

Due to the potential association between periodontitis and CVD, studies have been carried out to assess the hsCRP and IL-6 levels as possible risk factors of CVD in patients with CP. In the present study, we used the Sprague-Dawley rat model with CP and/or hyperlipidemia (HL) to simulate clinical periodontitis individuals. The aim of the study was to investigate the effects of nonsurgical periodontal therapy on the carotid artery, and the serum hsCRP and IL-6 levels in CP rats with or without HL.

Materials and methods

Animal grouping and model

A total of 29 male Sprague-Dawley rats (Beijing HFK Bioscience, Beijing, China) matched for age and weight (6-weeks-old, and between 180 to 200 g, respectively) were used (Certificate of quality SCXK [Jing], 2009-0004). The study protocol was approved by the Animal Experiment Review Committee at the Shanxi Medical University. All animals received humane care in accordance with the Guide for the Care and Use of Laboratory Animals[12]. After acclimatisation for 2 weeks, the rats were divided randomly into two groups: group A (CP group, n = 14); and group B (CP+HL group, n = 15). Groups A and B were further equally divided into groups A1/B1 (without periodontal intervention) and A2/B2 (with periodontal intervention). The animal models were established as previously described[13], as follows:

1. CP group – the rats were anaesthetised with an intraperitoneal injection of 5% chloral hydrate (Parasitology Laboratory of Shanxi Medical University, Shanxi, China) and placed on an operating table, with the head and limbs fastened. The first and second molars (M1 and M2) of both maxillary quadrants were chosen as the experimental teeth. The rats had a 0.2-mm-diameter orthodontic ligature twining with a 3-0 silk suture around the cervix of the experimental teeth. The silk suture was inoculated with a slurry containing *Porphyromonas gingivalis* ATCC 33277 (1×10^6 CFU/ml) once every 3 days for 15 weeks (*P. gingivalis* ATCC 33277 strain was kindly provided by Dr Sheng Hui YANG from the Oral Microbiological Laboratory, Beijing Stomatological Hospital, Capital Medical University, China). The rats were fed a regular chow diet[14]. The ligatures and sutures were removed after 15 weeks from the beginning of the treatment.
2. CP + HL group – after acclimatisation for 2 weeks, the rats were treated once with an intraperitoneal injection of vitamin D3 solution (7×10^5 IU/kg) (Shanghai General Pharmaceutical, Shanghai, China). The normal chow diet of the rats was then gradually replaced with a high-fat diet (HFD) consisting of 1.5% cholesterol, 0.2% pig bile salts, 5% homemade lard and 93.3% basic diet during the course of 2 weeks[15,16]. One rat was randomly chosen and euthanised after 15 weeks. The sections of carotid artery were stained with oil red-O (Sigma-Aldrich, St. Louis, MO, USA) to observe foam cell formation and lipid deposition, which showed the successful establishment of the model. Simultaneously, the periodontitis model was induced as mentioned above.

Periodontal interventions

In groups A1 and B1 the rats were not subjected to the periodontal intervention. The rats in groups A2 and B2 were subjected to nonsurgical periodontal treatment (including scaling and root planing). Two experimental teeth (maxillary first and second molars) from the same quadrant were treated each time and twice in total (Fig 1).

Periodontal assessment

Examination of clinical periodontal parameters was conducted 2 weeks after acclimatisation. Each experimental

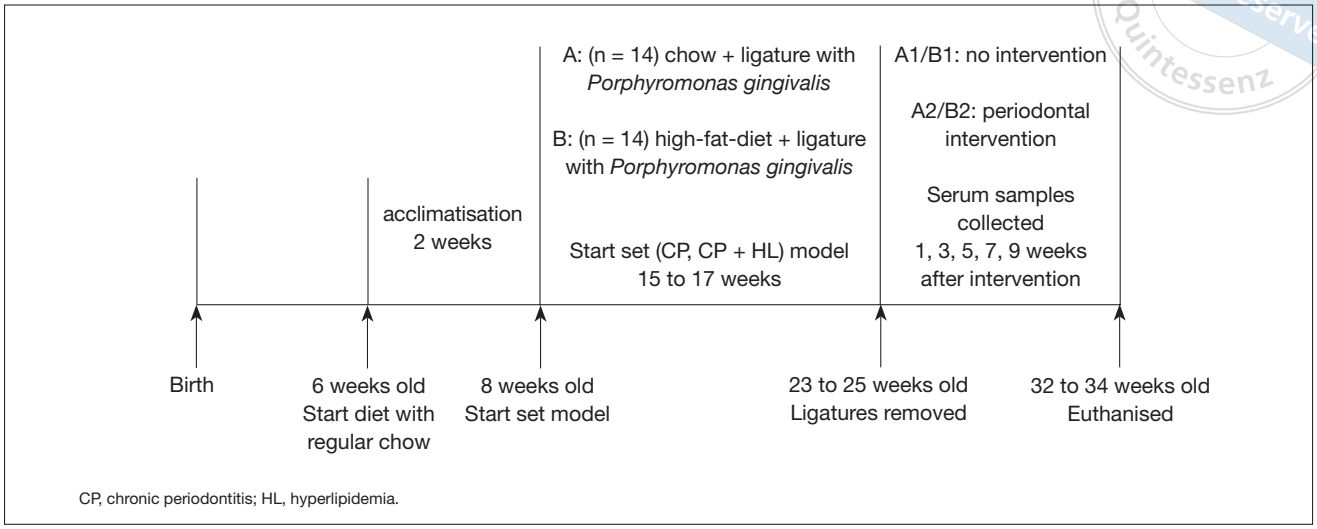

Fig 1 Animal grouping and experimental time schedule.

tooth was examined for probing depth, bleeding index (BI), and tooth mobility (Miller Class II or higher mobility). Clinical measurements (probing depth and recession) were recorded to the nearest millimetre using the Williams periodontal probe (Hu-Friedy, Chicago, IL, USA) by a masked and calibrated researcher[14]. Calibration was completed at two time points: pre-experiment and intra-experiment evaluation. The same researcher recorded the parameters every 2 weeks.

Enzyme-linked immunosorbent assay (ELISA)

The time interval between the first and second intervention was 2 weeks. All the rats were fasted for 12 hours prior to being anaesthetised with 5% chloral hydrate. A 1.5-ml-aliquot of blood was collected from the tail vein of each rat. The serum levels of hsCRP and IL-6 were assessed using the enzyme linked immunosorbent assay (ELISA) kits (Shanghai BlueGene Biotech, Shanghai, China), according to the manufacturer's instructions, 1 week before periodontal intervention;1 week after the first intervention; and 1, 3, and 5 weeks after the second intervention. The assays were performed in triplicates and repeated three times, with similar results each time.

Specimen collection

The rats were sacrificed by ketamine anaesthesia one week after the fifth blood sampling. The common carotid artery and its bifurcation (Fig 2) were dissected (approximately 1-cm long), the adventitial fascia was removed, and the specimens were washed with sterilised saline and fixed for 48 hours with 10% neutral buffered formalin. Thin sections (1.5-μm thick) were cut and stained conventionally with haematoxylin-eosin (HE) to observe the cellular composition of the carotid artery tissue. The maxilla was removed for periodontal lesion quantification.

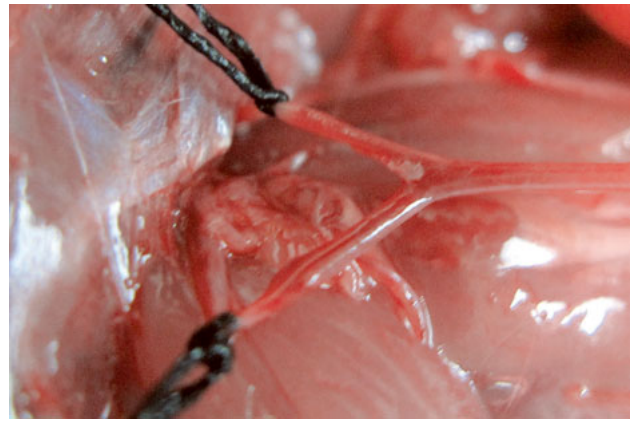

Fig 2 Common carotid artery and its bifurcation.

Statistical analysis

The data were analysed using the SPSS 13.0 statistical software (SPSS, Chicago, IL, USA). All data were presented as mean ± SD. The data were analysed by the student's t test (2-tailed) or one-way analysis of variance (ANOVA). The results with a P value < 0.05 were considered significant.

Table 1 Content of high-sensitivity C-reactive protein (hsCRP) in groups A1, A2, B1 and B2 before and after oral intervention (n = 7, μg/l).

Group	T1	T2	T3	T4	T5
A1	5.375 ± 0.622	5.257 ± 1.157[a]	6.442 ± 1.373[a]	6.505 ± 1.396[a]	7.219 ± 1.033[a]
A2	6.251 ± 1.024	7.173 ± 0.876[b]	8.934 ± 1.153[b]	6.369 ± 1.218	4.806 ± 0.940[b]
B1	7.741 ± 0.392[b]	8.156 ± 0.639[ab]	8.407 ± 0.460[ab]	9.271 ± 0.654[ab]	12.415 ± 0.592[ab]
B2	8.109 ± 1.274[b]	8.623 ± 1.233[b]	9.427 ± 1.275[b]	7.396 ± 1.222[c]	6.208 ± 1.504b[c]

[a]Compared with the same group of baseline, $P < 0.05$; [b]Compared with A1 group at the same time point, $P < 0.05$; [c]Compared with B1 group at the same time point, $P < 0.05$; T1, one week before the first periodontal intervention; T2, one week after the first periodontal intervention; T3-T5, 1, 3, 5 weeks after the second periodontal intervention.

Table 2 Interleukin 6 (IL-6) levels in groups A1, A2, B1 and B2 before and after oral intervention (n = 7, ng/l).

Group	T1	T2	T3	T4	T5
A1	22.905 ± 8.497	35.589 ± 9.368	40.750 ± 13.191	48.513 ± 17.605[a]	58.615 ± 11.359[a]
A2	24.514 ± 6.080	44.493 ± 11.746	62.254 ± 14.305[a]	48.891 ± 22.318[a]	28.644 ± 8.129[b]
B1	44.983 ± 8.471	46.084 ± 12.756	73.588 ± 27.893[ab]	100.726 ± 15.576[ab]	167.860 ± 33.719[ab]
B2	49.626 ± 6.577[b]	68.332 ± 8.856[bc]	94.709 ± 16.088[ab]	68.405 ± 20.745[c]	46.957 ± 9.862[c]

[a]Compared with the same group of baseline, $P < 0.05$; [b]Compared with A1 group at the same time point, $P < 0.05$; [c]Compared with B1 group at the same time point, $P < 0.05$; T1, one week before the first periodontal intervention; T2, one week after the first periodontal intervention; T3-T5, 1, 3, 5 weeks after the second periodontal intervention.

Results

Periodontal examination

The rats of groups A1 and B1 presented the typical symptoms of CP, including gingivitis, redness, bleeding upon probing, gingival recession, attachment loss, and class II or III tooth mobility. This was evident in the experimental teeth (maxillary first molar and second molar) in groups A1 and B1. Five weeks after the second periodontal intervention, the experimental teeth of rats from group B1 exhibited higher gingival recession, significant bleeding on probing (BI = 4), and tooth mobility (classes II and III). In groups A2/B2, the gingiva appeared pink and healthy with reduced gingival recession, BI = 1-2, and class I tooth mobility.

Expression levels of hsCRP and IL-6

The expression levels of serum hsCRP and IL-6 in the different groups before and after periodontal intervention is shown in Tables 1 and 2. The concentrations of serum hsCRP and IL-6 in the rats of groups A1 and B1 increased with time (Figs 3 and 4). The hsCRP level in the rats of group B1 was significantly higher (1.7 fold) than that in the rats of group A1 ($P < 0.001$) at all time points (12.415 ± 0.592 vs. 7.219 ± 1.033) at the end point (Fig 3). The IL-6 level in the rats of group B1 was significantly higher (2.9 fold) than that in the rats of group A1 ($P < 0.001$) at all time points (167.860 ± 33.719 vs. 58.615 ± 11.359) at the end point (Fig 4). In the periodontal intervention groups (A2 and B2), the hsCRP and IL-6 levels increased with time and reached the maximum levels 1 week after the second intervention (T3), and then gradually declined. At the end point, the hsCRP level in the rats of group A2 was lower than the baseline level (4.806 ± 0.940 vs. 6.251 ± 1.024), and the same trend was observed in group B2 (6.208 ± 1.504 vs. 8.109 ± 1.274) (Fig 3).The IL-6 level in the rats of group B2 was lower than the baseline level (46.957 ± 9.862 vs. 49.626 ± 6.577) (Fig 4). The hsCRP and IL-6 levels in the rats of groups A2 and B2 were also lower than that in the rats of groups A1 and B1 at the end point. The hsCRP level in the rats of group B2 was almost 50% of that of group B1 (6.208 ± 1.504 vs. 12.415 ± 0.592).The IL-6 level in the rats of group B2 was only 20% of that of group B1 (46.957 ± 9.862 vs.167.860 ± 33.719).

Pathological changes in the carotid artery

In the rats of group A1, the carotid artery wall was significantly thickened, a high number of foam cells was

formed and aggregated, and deranged elastic fibres were observed (Fig 5a). In group A2 rats, no obvious carotid artery wall thickening was observed, but a few foam cells and disorganised elastic fibres were present (Fig 5b). In group B1 rats, obvious atherosclerotic plaques emerged, and fibrous cap and calcium salt deposits were found along with inflammatory cell infiltration. Such altered vessel wall structures were observed in six rats of group B1 (Fig 5c). In group B2 rats, uneven thickened walls and inflammatory cells were noted on the surface of the carotid intima layer. Furthermore, the elastic fibres disappeared, but no obvious plaques were found (Fig 5d)

Discussion

The results obtained in this study showed that the serum hsCRP and IL-6 levels increased with the CP condition (a common potential source of low-grade inflammation) in Sprague-Dawley rat models. An additive effect on the expression of serum hsCRP and IL-6 was noted under conditions with periodontitis + HL, which can significantly increase the risk of atherosclerosis in rats. Pathologic changes in the rat carotid artery, such as atherosclerotic plaques, fibrous cap, and calcium salt deposits with inflammatory cells infiltration, were observed in the combined group (B1). Unexpectedly, such altered vessel wall structures were found in almost all the rats (6/7) in the combined group. Several studies have implicated oral infections, particularly periodontitis, as a risk factor of atherosclerotic CVD[17]. A recent large cross-sectional study showed that periodontitis is independently associated with atherosclerotic CVD[18]. As demonstrated, chronic inflammation, including bacterial infection, is involved in the development and progression of atherosclerotic vascular diseases[19,20]. On the other hand, increased periodontal infection has been found in patients with coronary heart disease[21]. Untreated periodontal disease is associated with high carotid intima-media thickness and high level of inflammatory markers, such as CRP[22]. Chronic periodontitis seemed to increase the level of CRP; thus, the control of periodontal infection by health professionals might improve cardiovascular health[23].

Some recent interventional trials have shown that periodontal treatment can decrease the markers of systemic inflammation[24]. Periodontal therapy triggers a short-term inflammatory response followed by a progressive and consistent reduction in systemic inflammation and an improvement in endothelial function. However, there is limited evidence to suggest that these acute and chronic changes will either increase or reduce CVD burden in individuals suffering from periodontitis

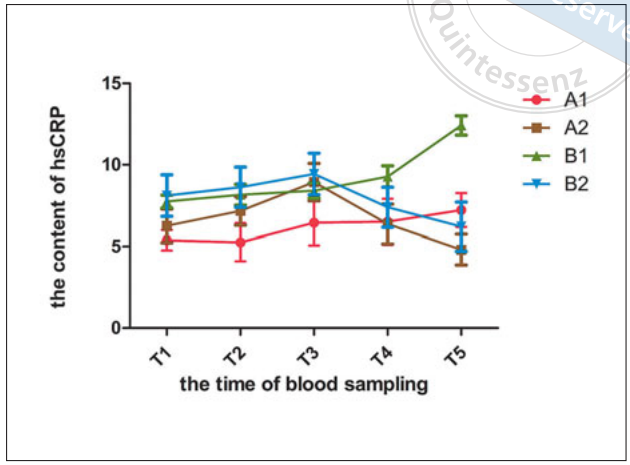

Fig 3 The level of hsCRP in group A1, A2, B1, B2 before and after periodontal intervention. A1: CP group (without periodontal intervention); A2: CP group (with periodontal intervention); B1: CP + HL group (without periodontal intervention); B2: CP + HL group (with periodontal intervention). The serum hsCRP level in groups A1 and B1 was elevated with time; they were significantly higher in group B1 than in group A1 ($P < 0.001$) at all time points. The hsCRP level in groups A2 and B2 increased with time and reached the maximum level at one week after the second intervention, and then gradually decreased. T1: one week before the first periodontal intervention; T2: one week after the first periodontal intervention; T3-T5: 1, 3, 5 weeks after the second periodontal intervention.

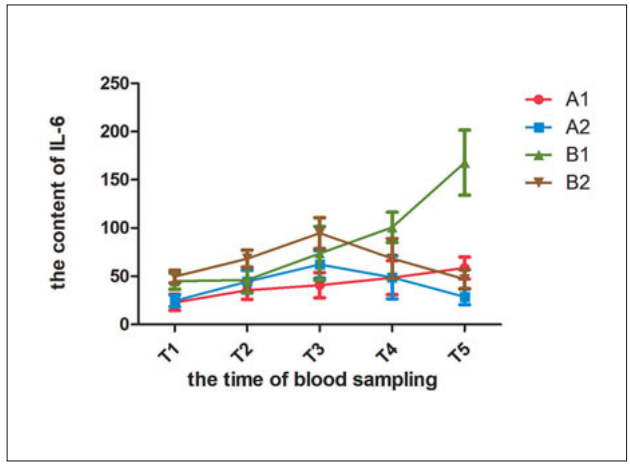

Fig 4 The levels of IL-6 in group A1, A2, B1, B2 before and after periodontal intervention. A1: CP group (without periodontal intervention) A2: CP group (with periodontal intervention); B1: CP + HL group (without periodontal intervention); B2: CP + HL group (with periodontal intervention). The serum IL-6 level in groups A1 and B1 was elevated with time; they were significantly higher in group B1 than in group A1 ($P < 0.001$) at all time points. The IL-6 level in groups A2 and B2 increased with time and reached the maximum level at one week after the second intervention, and then gradually decreased. T1: one week before the first periodontal intervention; T2: one week after the first periodontal intervention; T3-T5: 1, 3, 5 weeks after the second periodontal intervention.

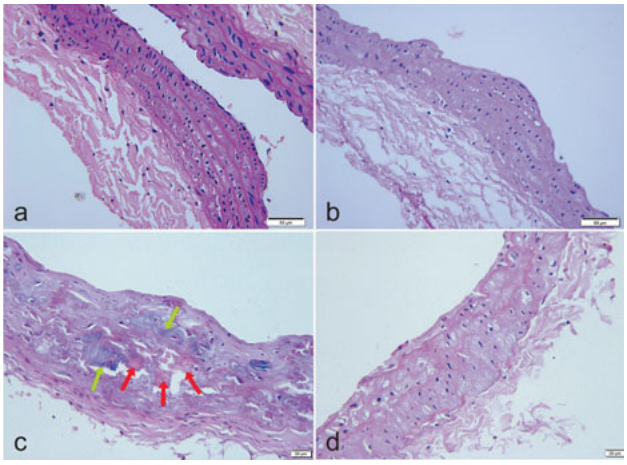

Fig 5 Pathological changes of vessel walls of the carotid artery (haematoxylin-eosin [HE] x 200. (a) Chronic periodontitis (CP) group (without periodontal intervention): the carotid artery wall was significantly thickened, a high number of foam cells formed and aggregated, and disordered elastic fibres were found; (b) CP group (with periodontal intervention): no obvious carotid artery wall thickening was observed; (c) Chronic periodontitis (CP) + hyperlipidemia (HL) group (without periodontal intervention): obvious atherosclerotic plaques (red arrows) emerged and calcium salt deposits (green arrows) were found along with inflammatory cell infiltration; (d) CP + HL group (with periodontal intervention): no obvious plaques were found.

in long term[25]. Results of intermediate surrogate markers can only be available from human clinical studies, and no direct evidence of vascular atherosclerotic disease is available to support these speculations due to ethical reasons. Animal experiments enable collection of specimens after interventions. The results of the present study showed that a periodontal intervention on the rat periodontitis model significantly increased the level of serum hsCRP and IL-6 1 week after the treatment, especially in the combined group. Interestingly, the level of hsCRP and IL-6 in the intervention groups reached the maximum after intervention, and then gradually declined with time. This suggests that nonsurgical periodontal treatment might have a beneficial effect in minimising the development of atherosclerosis and improving the structure of carotid vessels. Previously, it has been reported that individuals who did not respond well to the periodontal treatment had increased risk of CVD, indicating that successful periodontal treatment might influence the progression of subclinical CVD[26].

Several clinical studies have shown that periodontal treatment improves endothelial function, reduces the expression of biomarkers of atherosclerotic disease, and reduces the thickness of carotid intima-media, especially in those suffering from CVD[27,28]. Recently, a study indicated that patients with metabolic syndrome and chronic periodontitis, treated with nonsurgical periodontal treatment, may be at decreased risk for atherosclerosis and CVD[29]. Oral hygiene instruction and mechanical periodontal instrumentation were effective in promoting the reduction in carotid intima-media thickness six months after treatment[30]. In addition, it has been reported that advanced periodontal therapy lowers the rate of CVD, especially myocardial infarction and heart failure[31]. These studies suggest that the periodontal treatment might reduce the risk of atherosclerosis. In another study, the rate of vascular events significantly increased during the first four weeks after invasive dental treatment (incidence ratio, 1.50 [95% CI, 1.09-2.06]) and gradually returned to the baseline rate within six months; however, the absolute risks were minimal, and the long-term benefits on vascular health will probably outweigh the short-term adverse effects[32].

In the present study, treatment of periodontitis was associated with reduced serum hsCRP and IL-6 levels and improved the carotid artery vascular function. This implies that periodontal interventions might result in acute, short-term systemic inflammation, which might increase the risk of atherosclerosis, especially in periodontitis rats with HL. However, the effect on the improvement of carotid artery vascular is beneficial over time. Our findings support those of D'Aiuto et al[33], who conducted a human clinical intervention study. Their results showed that patients with severe periodontitis have significantly higher levels of inflammatory factors including CRP and lower flow-mediated dilatation 24 hours after intensive nonsurgical (scaling and root planing [SRP]) than those of patients without SRP. Interestingly, greater flow-mediated dilatation was observed in the SRP group than in the non-SRP group 60 and 180 days after therapy. Their study clearly demonstrated that intensive periodontal treatment results in acute, short-term systemic inflammation and endothelial dysfunction. However, the benefits in oral health were associated with the improvement in endothelial function 6 months after therapy.

The results of the present study suggest that nonsurgical periodontal treatment results in acute, short-term systemic inflammation and that it is more beneficial in the long term[34] as it improves carotid artery integrity. This study might provide important reference values for the clinical setting and avoid the exacerbation and death of patients with HL caused by oral intervention.

Conflicts of interest

The authors reported no conflicts of interest related to this study.

Author contribution

Drs Miao Miao WANG and Yong ZHAO contributed to the work equally, participated in collecting data and drafted the manuscript; Drs Chong WANG, Xue Xue SHI and Jin Hua GAO performed the clinical treatment and statistical analysis; Dr Xiu Yun REN designed and supervised the study.

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