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Original Article

Preliminary study on the involvement of platelets in mouse experimental periodontitis

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Abstract *Background/purpose:* Although some studies have taken an interest in the participation of platelets in periodontitis, so far, we know very little about the roles of platelets in periodontitis. The objective of this study is to explore the involvement of platelets in the development of experimental periodontitis in mice.
Materials and methods: Twenty C57BL/6 male mice were used for this study. Experimental periodontitis models of mice were constructed by ligating for 1, 3, 7, and 14 days, respectively. Morphological changes in the alveolar bone were assessed by micro-computed tomography (Micro-CT). The gingival crevicular fluid samples of ligation sites were collected and stained by immunocytochemistry. Immunohistochemistry was used to detect platelets infiltration in gingival tissues of mice.
Results: The results of Micro-CT showed that with the extension of ligation time, alveolar bone resorption increased, suggesting that the experimental periodontitis models were established. Immunochemical staining showed that there were almost no platelets in the gingival crevicular fluid of mice ligated for 1 and 3 days. And at 7 and 14 days of ligation, a large number of platelets

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were present in the gingival crevicular fluid and formed complexes with neutrophils. And with the extension of ligation time, the extent of platelet infiltration increased in mice gingival tissues.
Conclusion: Platelets were infiltrated increasedly in the gingival sulcus and gingival tissues following the experimental time, and may participate in the development of mouse experimental periodontitis.

Introduction

Periodontitis is a chronic inflammation of the periodontium. Most destruction of tooth-supporting tissues is due to the host's immune response to bacterial infection. Inflammatory cells and cytokines play important roles in host responses during the occurrence and development of periodontitis. The previous studies of the immunopathology of periodontitis mainly focused on neutrophils, B cells, macrophages, etc.[1—3]

Platelets are small pieces of cytoplasm that are shed from mature megakaryocytes in the bone marrow, without nuclei. Platelets are generally considered to be the key mediators of blood clotting. Substantial evidence has revealed that platelets are also inflammatory cells. During the immunoinflammatory responses, such as immune recognition and inflammatory damage, platelets cannot be ignored. Platelets can not only directly interact with other inflammatory cells, such as neutrophils, endothelial cells, etc., but also indirectly regulate other inflammatory cells by secreting immune mediators.[4—7] In addition, the inflammatory response of platelets could emerge locally in platelet activation and aggregation, and can also occur systemically through the secretion of a large number of inflammatory mediators.[8] In some chronic inflammatory diseases, relevant indicators such as mean platelet volume (MPV) and platelet distribution width (PDW) can reflect disease activity and the effect of anti-inflammatory treatment.[9,10] Studies have found that platelets play an important role in the occurrence of atherosclerosis (AS), rheumatoid arthritis (RA), inflammatory bowel disease (IBD), and other diseases.[11—15]

Preliminary studies have found platelets may play an important role in periodontitis.[16—18] A large number of platelet-neutrophil aggregates in the inflammatory gingiva of patients with aggressive periodontitis.[18] However, it is unclear how platelets take part in the development of periodontitis. In addition, how platelets migrate to gingiva during the development of periodontitis is unknown.

Compared with healthy subjects, there were more platelets in the gingival crevicular fluid of patients with periodontitis and gingivitis, and more platelet factor 4 (PF4) was detected in the gingival crevicular fluid (GCF) of patients with periodontitis,[19] which is the first report on platelets in the GCF. While it is unclear how platelets migrate to the gingival sulcus and what role to play in the GCF.

In this study, we attempted to explore the infiltration of platelets in the GCF and gingival tissues in the experimental periodontitis mice.

Materials and methods

Establishment of experimental periodontitis models in mice

Seven-week-old C57BL/6 male mice, weighing nearly 22 g, were used for this study. Mice were randomly divided into 4 groups (n = 5 per group): 1-day ligation (L1), 3-day ligation (L3), 7-day ligation (L7), and 14-day ligation (L14), respectively. After intraperitoneal injection of anesthesia, sterile silk ligatures (5—0) (Jinhuan Medical, Shanghai, China) were placed at the cervical areas of the bilateral maxillary second molars and knotted on the palate sides. White dried cotton was used to check for bleeding several times. In case of bleeding, the ligation was stopped and performed again the next day. After 1, 3, 7, and 14 days of ligation, the silk ligatures were removed, and then the mice were sacrificed. Maxillae were dissected carefully and fixed in 4% paraformaldehyde solution for 48 h for further analysis. All animal experimental procedures were approved by the Animal Welfare Ethics of Peking University Biomedical Ethics Committee (LA2018252).

Micro-CT analysis

The mice maxillae were scanned using the micro-computed tomography (Micro-CT) system. The distance between the cementoenamel junction (CEJ) and the alveolar bone crest (ABC), the bone volume/tissue volume (BV/TV) ratio, and bone mineral density (BMD) in the region of interest of the maxillary second molar were analyzed with Inveon Research Workplace software (Siemens, Berlin, Germany).

Collection and processing of the gingival crevicular fluid from mice

Two ligature threads were placed in an eppendorf tube with 400ul PBS buffer (containing protease inhibitors). If blood contamination occurs during taking the ligated thread, the thread was discarded, and later, another mouse was supplemented to ensure the number of samples. The tubes were vibrated at 1.5 A for 10 min. Then, the GCF samples were centrifuged by the cytospin (Thermo Fisher Scientific, Waltham, MA, USA) at 1000 rpm for 3 min. Two smears were made per 400ul gingival crevicular fluid sample and fixed with 4% PFA for 30 min. Then Wright-Giemsa staining was used to exclude blood contamination again, and platelets were detected by immunocytochemical staining.

Immunocytochemical staining

The GCF smears were incubated with 3% H_2O_2 for 15 min. To prevent non-specific binding, the smears were blocked with 10% goat serum (Zsbio, Beijing, China) for 30 min. Then incubated with rabbit monoclonal anti-CD41 (1:200, Abcam, Cambridge, UK) overnight at 4 °C, followed by incubation with goat anti-rabbit IgG (Zsbio) for 30 min at 37 °C before staining with 3,3′-diaminobenzidine (DAB) (Zsbio) for 3min. The nuclei were counterstained with hematoxylin.

Immunohistochemistry staining

Mice maxillae were decalcified in 10% EDTA (pH 7.4) for two weeks. The sections of mice maxillae were dehydrated in a series of alcohol and embedded in paraffin. Paraffin blocks were cut into 5-um-thick mesiodistal serial sections. Paraffin sections undergo dewaxing, hydration, and high-temperature antigen repair. Then the sections were incubated with 3% H_2O_2 and blocked with 10% goat serum. Tissue sections were stained with antibodies against CD41 (1:200, Abcam) overnight at 4 °C, and incubated with secondary antibody (Zsbio) at 37 °C. The positive staining was visualized with DAB (Zsbio) and counterstained with hematoxylin.

Statistical analysis

Statistical analysis was performed using SPSS 27.0 software. Data were presented as mean ± SD. The statistical significance of differences among groups was determined by independent two-tailed Student t tests or one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

Results

The experimental periodontitis models were established successfully

The three-dimensional reconstruction and sagittal view of Micro-CT revealed the absorption of alveolar bone of the maxillary second molars (Fig. 1a and b). For the distance of CEJ-ABC and BV/TV, there was no significant difference between 1 and 3 days after ligation, but they changed significantly on 7 days after ligation. The distance of CEJ-ABC reached the maximum (Fig. 1c), and BV/TV reached the minimum (Fig. 1d) on 14 days after ligation. For BMD, this index decreased with the extension of ligation time and reached the minimum at 7 days (Fig. 1e). These results indicated that the mice experimental periodontitis models were established successfully.

Aggregation of platelets and neutrophils in mouse gingival crevicular fluid

As shown in Fig. 2, a small number of neutrophils were observed in the GCF of mice on day 1 and 3 of ligation, bacteria were mostly dispersed and not aggregated, and

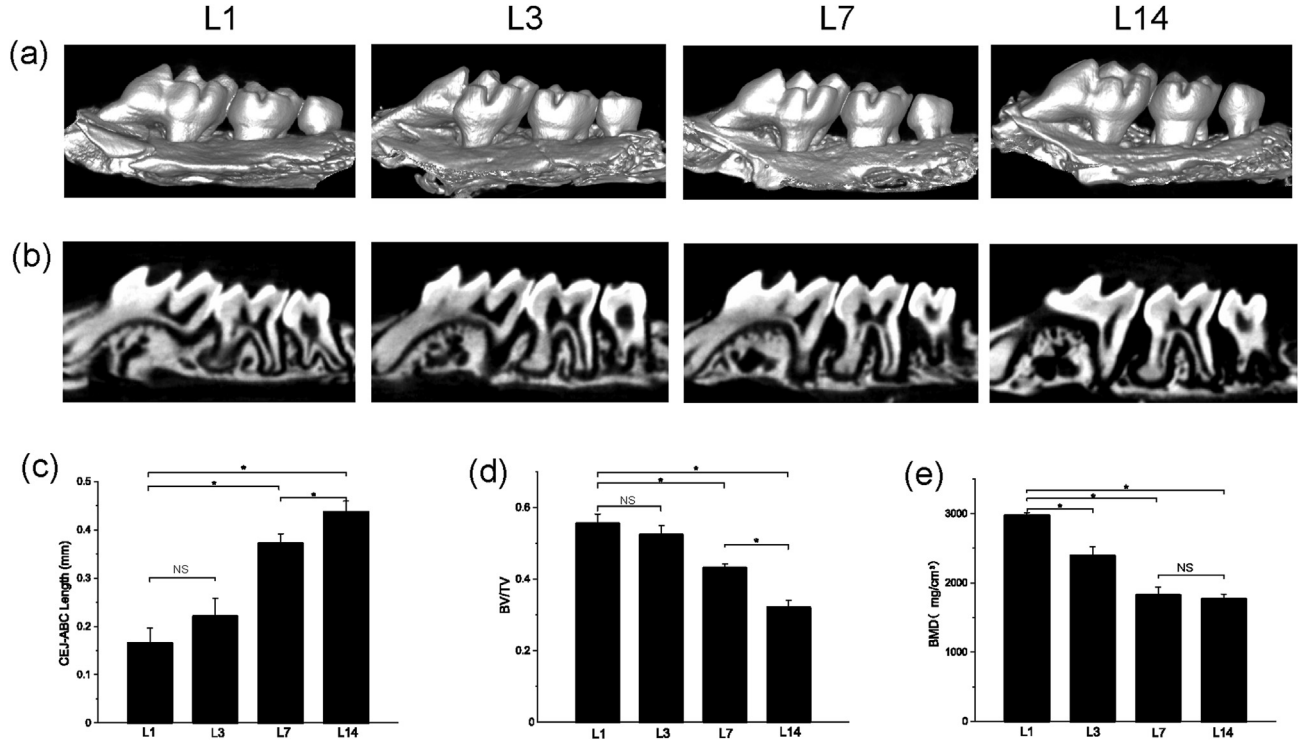

Figure 1 Experimental periodontitis models of mice were assessed by Micro-CT. a) Representative images showed a three-dimensional horizontal view of the maxilla. The groups' names are abbreviated as follows: 1-day ligation group (L1), 3-day ligation group (L3), 7-day ligation group (L7), and 14-day ligation group (L14). b) Sagittal perspectives of the maxilla were presented. Quantification analysis of the distance of CEJ-ABC (Fig. 1c), BV/TV (Fig. 1d), and BMD (Fig. 1e). *: $P < 0.05$.

few platelets were observed. At 7 and 14 days of ligation, a large number of neutrophils (lobulated nuclei) and clumps of bacteria (cocci and rod-shaped) were observed. Platelets ($CD41^+$, brown) adhered to neutrophils, and bacterial clumps surrounded by neutrophils and platelets in GCF.

Platelet infiltration in the gingival tissue of mice

The immunohistochemical staining of the gingival tissues of mice revealed that, after one day of ligation, the gingival sulcus epithelium and the junctional epithelium were intact, and there was no infiltration of leukocytes or platelets. In the 3-day ligation group, the junctional epithelium was destructed partially, periodontal pockets were formed, platelets were infiltrated in gingival tissues, and platelets mostly adhered to the blood vessel wall. In the 7-day ligation group, the gingival sulcus epithelium was incomplete, the periodontal pocket was deeper, and a large number of leukocytes and platelets were infiltrated diffusely in the gingival epithelium and connective tissues. While, the infiltration of platelets in the 14-day ligation group was less than that of the 7-day ligation group (Fig. 3).

Discussion

In this study, the experimental periodontitis models in mice were constructed using the classic silk thread ligation method.[20] The advantage of the ligature method is that periodontitis can be initiated at a definite time and induce significant host inflammatory responses in a specific location within a short period.[21] In this study, we detected the extent of platelet infiltration in gingival tissue increased with prolonged ligation time. At 3 days of ligation, platelets adhered to the endothelium of the gingival microvessels. Platelet-endothelial cell interaction is critical for initiating the leukocyte recruitment cascade. When the endothelium is damaged, the level of prostaglandin and ADP hydrolase secreted by endothelial cells decreases, the adhesion of platelets is enhanced, and the GPIbα expressed by platelets binds to selectins and vWF on the surface of endothelial cells.[22] Platelets express P-selectin, which binds to circulating leukocytes and recruits a large number of leukocytes to the sites of endothelial injury, leading to an inflammatory response.[23] At 7 days of ligation, platelets and leukocytes diffuse infiltrated in the gingival tissues, and a significant proportion of platelets aggregated with leukocytes. The interaction of platelets with leukocytes is the key to the progression of inflammation. Platelet P-selectin specifically binds to PSGL-1 on leukocytes, and then activated platelets can release a range of inflammatory mediators, such as platelet-activating factor (PAF), interleukin-1β, and chemokines.[5] On the other hand, the platelet-neutrophil aggregates are important for neutrophil activation and migration, resulting in inflammatory damage.[24] Therefore, the observation of immunochemistry staining

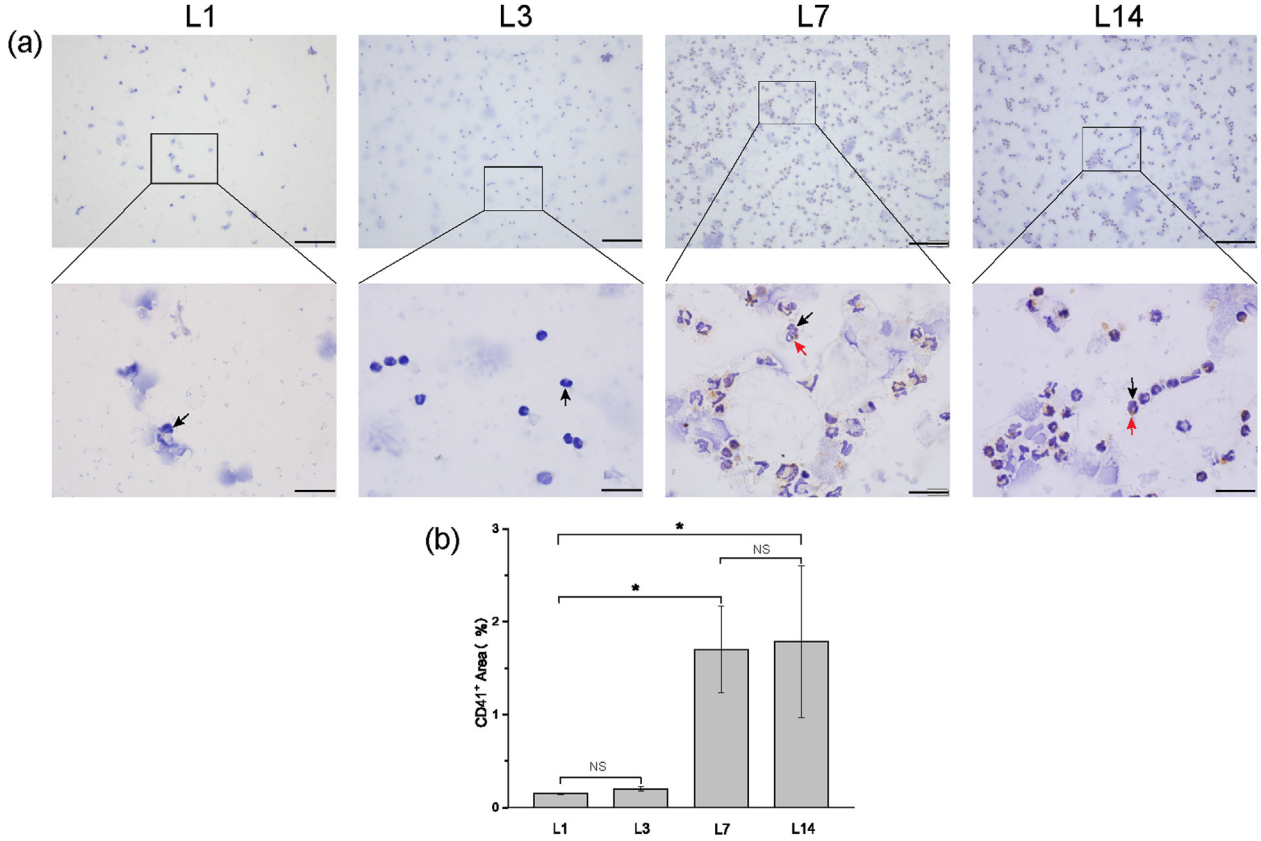

Figure 2 Platelets were present in the gingival crevicular fluid at the mice periodontitis sites. a) Immunocytochemical staining was used to observe platelets in the GCF of mice ligated on different days. Red arrows indicate platelets. Black arrows indicate neutrophils. Bar = 100 μm (above), 20 μm (below). b) Quantification analysis of the level of platelets in different groups. *:$P < 0.05$.

suggests that platelets may be involved in the initiation and progression of periodontitis. Our results showed that the extent of platelet infiltration in gingival tissues at 14 days after ligation was less than that of 7 days, although the platelet level in GCF was still high at 14 days. One study about mouse experimental periodontitis model has found that the expression levels of chemokines and genes associated with innate immunity peaked at 9 days after ligation, then declined at 15 and 18 days.[21] The expression of myeloperoxidase was also highest at 9 days of ligation and decreased at 15 days of ligation. This evidence suggests that experimental periodontitis may undergo innate immunity and adaptive immunity successively. Therefore, it is possible that in this study, innate immunity mainly occurs in the first 7 days. Platelets are similar to neutrophils and mainly play the innate immune effect. Later, with the development of inflammation, the adaptive immunity is dominant. B cells and plasma cells dominate in the tissues, and platelets infiltration decreases. However, further research is needed to explore the phenomenon.

We improved the previously reported method[25] to collect and process the gingival crevicular fluid of mice, the modified method could be easily used to find platelets in GCF. In this study, we observed that, after 7 and 14 days of ligation, platelets adhered to neutrophils, forming platelet-neutrophil aggregates in GCF of mice. This suggests that platelets may be involved in the immune defense of mouse gingival crevicular fluid. Platelet is also one kind of immune cells and expresses multiple TLRs, including TLR1, TLR2, TLR4, TLR6, TLR7, and TLR9.[26–29] Consequently, platelets

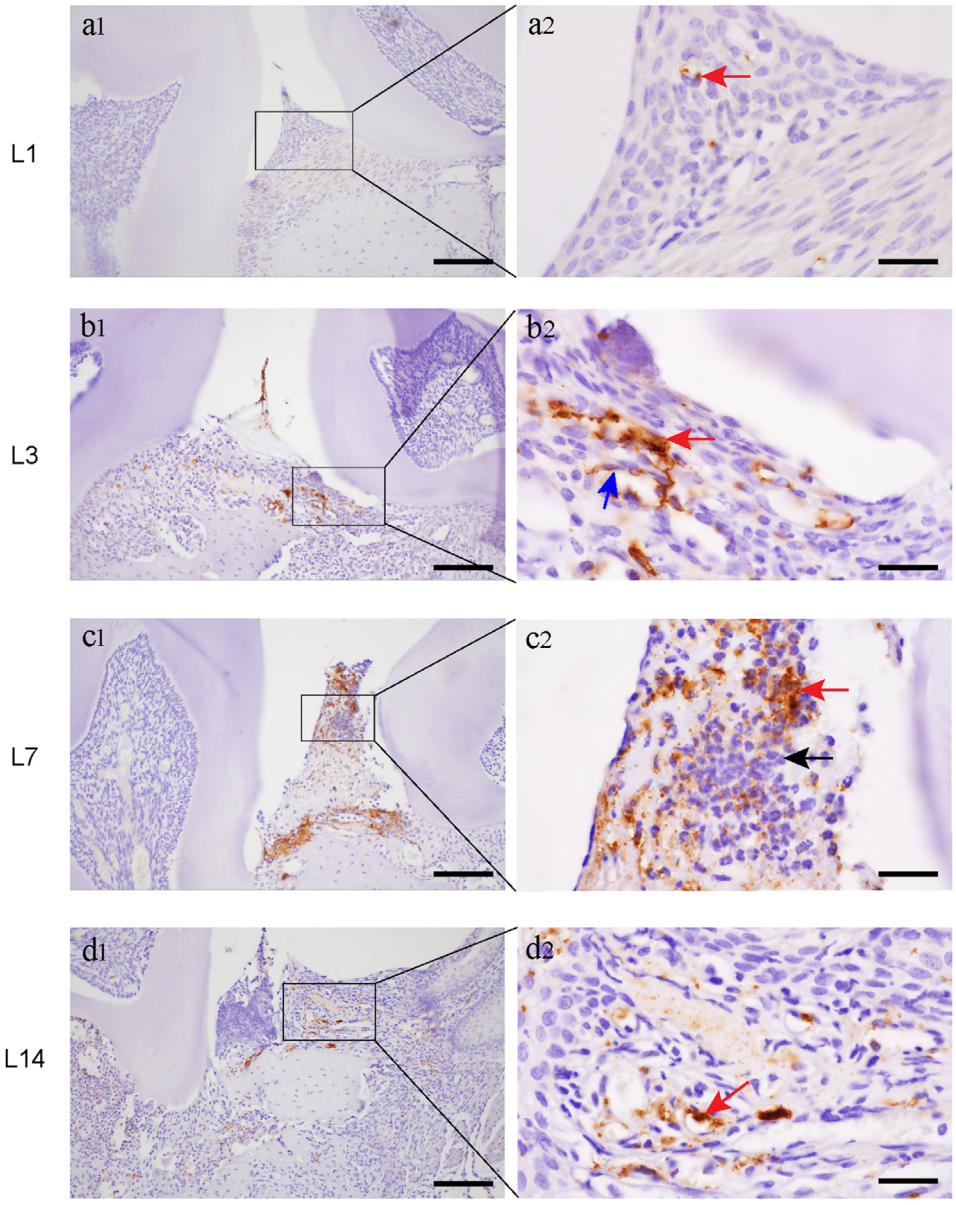

Figure 3 Platelet infiltration in different stages of experimental periodontitis. a) Immunohistochemical staining showed the extension of platelet infiltration as the ligation time prolonged. Bar = 100 μm (a1, b1, c1, d1), 20 μm (a2, b2, c2, d2). Red arrows indicate platelets. Black arrows indicate leukocytes. Blue arrows indicate blood vessel walls.

can interact with other immune cells involved in both the innate and adaptive immune response. Reportedly, platelets are capable of forming complexes with neutrophils,[30] which may be the result of platelets exposed to LPS. In sepsis models, platelets can pool *Escherichia coli* into bundles, and platelet-bound bacterial clusters can resist shear forces, promoting the activation of neutrophils and the formation of NETs.[31] Some evidence suggests that platelets have a direct inhibitory effect on microbial growth and proliferation. It has been found that platelets can directly inhibit the growth of *Staphylococcus aureus* by damaging bacterial DNA and blocking cell division.[32] Also, activated platelets could release a variety of bioactive molecules, such as PF4, β-TG, HMGB1, etc,[33,34] and exert direct antibacterial effects through degranulation and release of antibacterial peptides. However, the specific immune effect of platelets in the GCF needs to be further studied.

Wright-Giemsa staining is commonly used in blood smears.[35,36] It can easily display all kinds of blood cells (red blood cells (above 90%), white blood cells, and platelets). If the number of red blood cells on a GCF smear is less than 5%, it is considered that there is no blood contamination. In the past, it was generally considered that the platelets outside the blood vessels in tissues were only the result of exudation from the blood vessels due to endothelial injury. However, in this study, after excluding blood contamination by Wright-Giemsa staining, we detected platelets in the gingival crevicular fluid samples of mice. And during the development of periodontitis, capillaries increased, and the extent of platelet infiltration in tissues gradually increased. We speculate that platelets may, like neutrophils, undergo a migration process to reach the gingival sulcus. Of course, more studies are needed to verify this migration, especially real-time dynamic studies. We also need to further explore how platelets pass through the endothelium of blood vessels, whether the migration of platelets is like neutrophils driven by certain chemoattractants, and whether the migration process is related to neutrophils. Pitchford et al. found that platelets migrated extravascularly in response to a sensitizing allergen, and detected platelets in the lung parenchyma of mice.[37] It was observed in serial ultrathin sections that within 15 min of intradermal injection of 10^{-5} M N-formylmethionylleucine phenylalanine (fLMP) in guinea pigs, platelets adhered to the venous endothelium surface, after which platelets migrate to extravascular space.[38] In vitro study, platelets were able to migrate across endothelial cells and the Transwell membrane upon chemotaxis by stromal cell-derived factor 1 (SDF-1).[39] The above evidence suggests that platelets have the ability to migrate and SDF-1 may be one chemotactic molecule for platelet migration. However, there are fewer reports about platelet migration. More studies are needed to confirm its migration ability.

As far as we know, this is the first cytological study of mouse gingival crevicular fluid. In terms of methods, the collection and processing method of gingival crevicular fluid makes the visualization of cells in GCF easy and reproducible. The limitation of this study: the role of platelets in the gingiva is currently mainly focused on the observation of the phenomenon, and the corresponding molecular mechanism needs to be further explored. What's more, dynamic observation of platelet migration should be made, and further experiments of removing platelets or impairing platelet functions may provide more evidence.

In conclusion, this study is the first to find the presence of platelets in the GCF of experimental periodontitis mice. With the extension of ligation time, the extent of platelet infiltration increases, and platelets may undergo a migration process to reach the gingival sulcus. These findings enhance our understanding of the pathogenesis of periodontitis.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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