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Pro-inflammatory cytokine interleukin-6-induced hepcidin, a key mediator of periodontitis-related anemia of inflammation

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Abstract

Objectives: To investigate whether anemia of inflammation (AI) occurs in periodontitis patients and to further explore underlying pathogenesis of periodontitis-related AI by an experimental periodontitis model.

Background: Previous studies have reported periodontitis patients could show a tendency toward AI. However, the relationship between periodontitis and AI remains unclear, and the related pathological mechanisms have not been identified.

Materials and Methods: Periodontal clinical parameters, inflammatory markers, and anemia-related indicators were compared between 98 aggressive periodontitis (AgP) patients and 103 healthy subjects. An experimental periodontitis model was induced by ligature placement in mice. The changes in mice inflammatory markers, anemia indicators, hepcidin mRNA expression, and serum hepcidin concentrations were measured. Human and mouse liver cells were treated with interleukin-6 (IL-6) for analyzing the changes in hepcidin expression based on mRNA and protein levels.

Results: AgP patients exhibited higher white blood cell counts, IL-6, and C-reactive protein. Adjusted linear regression analyses showed correlations between AgP and decreased hemoglobin (HGB) and hematocrit (HCT). The ligature-induced periodontitis caused systemic inflammation and elevated IL-6 levels. Lower red blood cell counts, HGB, and HCT were detected, whereas the levels of hepcidin mRNA expression and serum hepcidin concentrations increased. The treatment of hepatocytes with IL-6 induced both hepcidin mRNA expression and hepcidin secretion.

Conclusions: Systemic inflammation induced by periodontitis leads to an increased risk for AI. IL-6-induced hepcidin could play a central mediator role and act as a key pathologic mechanism. Our results demonstrate periodontitis may be considered as an additional inflammatory disease contributing to the development of AI.

KEYWORDS
anemia of inflammation, experimental periodontitis, hepcidin, interleukin-6, periodontitis

Yalin Zhan and Jianxia Hou have contributed equally to the work.

1 | INTRODUCTION

Periodontitis, caused by bacterial infection, is one of the most common chronic inflammatory diseases affecting tooth-supporting tissues.[1] Depending on the population, 18% to 60% of young adults and up to 96% of older adults will likely be affected by periodontal diseases.[2] The pathogenic microorganisms and their products evoke immune-inflammatory responses in host tissues, resulting in elevated numbers of white blood cell (WBC) counts[3] as well as increased levels of C-reactive protein (CRP)[4] and inflammatory cytokines.[5] Evidence has verified that periodontitis is a systemic inflammatory condition.[6,7] Research has shown a definite relationship between periodontal diseases and systemic diseases such as diabetes mellitus,[8] cardiovascular disease,[9] and rheumatoid arthritis.[10] Poor periodontal condition has a significant effect on systemic health.

Anemia of inflammation (AI), also referred to as anemia of chronic disease, is the second most prevalent type anemia affecting hospitalized and chronically ill patients.[11] AI is a mild-to-moderate type of anemia that develops in association with inflammatory diseases that cause prolonged immune activation and systemic inflammation.[12] These diseases, such as acute and chronic infection, autoimmune disease, cancer, organ failure, trauma, and other causes of inflammation, have been recognized to contribute to anemia.[13] Periodontitis is also an inflammatory disease of host immune responses against bacterial infections, which results in the destruction of periodontal supporting tissues and adverse effects on systemic health.[14] The association between anemia and periodontitis has been studied since the early 20th century, when Lainson first implicated anemia as a systemic cause of periodontitis.[15] Moreover, a few studies have reported that patients with periodontitis also exhibited sign of anemia.[16-18]

AI is stimulated by inflammation and a systemic iron disorder in which iron is sequestered in reticuloendothelial cells. In such cases, intestinal iron absorption is decreased, and the limitations of iron delivery to maturing erythrocytes impair hemoglobin synthesis and eventually result in iron-restricted erythropoiesis.[13] However, the related pathological mechanisms underlying the relationship between periodontitis and AI remain unclear; thus, further research is needed to elucidate the pathogenesis of periodontitis-related AI.

Therefore, the present study aims to investigate whether periodontitis patients have a tendency of arising AI and the relationship between periodontitis and AI and further explore the possible pathogenesis of periodontitis-associated AI.

2 | MATERIAL AND METHODS

2.1 | Ethics statement

The study was approved by the Ethics Committee of Peking University Health Science Center (Approval No. RB00001052-08010). Informed consent was obtained from all subjects.

2.2 | Human subject collection

For this study, 98 patients with aggressive periodontitis (AgP) were recruited from the Department of Periodontology, Peking University School and Hospital of Stomatology. The diagnosis of the disease was based on diagnostic criteria proposed by 1999 International Workshop for a Classification of Periodontal Diseases and Conditions[14] as follows: systematically healthy with the onset of periodontal destruction at <35 years of age; at least eight teeth with probing depth (PD) >5 mm and attachment loss (AL) >3 mm; and radiographic evidence of interproximal bone loss. According to the new classification of periodontal diseases and conditions adopted in 2018,[19] the diagnoses were periodontitis stage III or IV.

In addition, 103 healthy subjects were selected from the staffs and students of Peking University School and Hospital of Stomatology with the following inclusion criteria: (1) probing depth (PD) ≤3 mm, (2) percentage of sites with bleeding on probing (BOP) <10%, (3) no attachment loss (AL), and (4) no bone loss on radiographs.

In addition, the exclusion criteria for all subjects were (1) current or previous smoker, (2) presence of systemic disease or pregnancy, (3) history of taking iron or other drugs to treat anemia, and (4) history of periodontal therapy or antimicrobial therapy or usage of any medication (including oral contraceptive drugs) within the previous six months.

2.3 | Clinical examination

All subjects completed a questionnaire and underwent a full-mouth clinical periodontal examination, including PD and AL, in which William's periodontal probe was employed on six points on each tooth. In addition, the bleeding index (BI) was recorded for each tooth. All examinations were performed by systematically trained and calibrated clinical periodontists.

2.4 | Blood collection and hematological analyses

Fasting blood samples were obtained from each subject between 08:00 and 11:00 local time by venipuncture using ethylenediaminetetraacetic acid (EDTA)-containing and coagulant-containing tubes, and were sent to testing laboratories within 30 min of collection. The complete blood cell and biochemical analyses were conducted by a technician blinded for the case status using a calibrated automated hematology analyzer (Sysmex) and a biochemistry automatic analyzer (Model 7180; Hitachi), respectively. Biochemical analyses were conducted for WBC counts, red blood cell (RBC) counts, hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). The concentrations of interleukin-6 (IL-6) and CRP were measured using enzyme-linked immunosorbent assay (ELISA) kits (IL-6; R&D Systems; CRP; Diagnostic System Laboratories, respectively) according to the manufacturer's instructions.

2.5 | Histology and immunohistochemistry analyses of human gingival tissues

Gingival tissues were harvested using horizontal incisions made 2 mm apically to the gingival margin when extracting third molars from five healthy volunteers and when extracting teeth with severe AgP from five patients. The gingival biopsies were fixed in 4% paraformaldehyde and were processed in paraffin for routine histology, and 5-μm sections were stained with hematoxylin and eosin (H&E). The localization of IL-6 in gingival tissues was detected by immunocytochemistry using a rabbit monoclonal antibody (Abcam) according to the manufacturer's instructions. Immunoreactive score (IRS) with modification was used to evaluate immunohistochemical staining of IL-6 in the gingival tissue. Briefly, IRS = staining intensity (SI) × percentage of positive cells (PP). The SI is defined by values of 0: negative; 1: weak; and 2: moderate; 3: strong. The PP values are 0: negative; 1: 0%–10% positive cells; 2: 10%–50% positive cells; 3: 50%–75% positive cells; and 4: 75%–100% positive cells. Five visual fields for each specimen were chosen for the IRS evaluation and statistical analyses.

2.6 | Establishment of ligature-induced experimental periodontitis in mice

The study protocol was approved by the Experimental Animal Welfare Ethics Branch of Peking University Biomedical Ethics Committee (Protocol LA2013-32). The animal experiments were conducted in the Animal Laboratory, Peking University School and Hospital of Stomatology. A total of 20 male C57BL/6 mice 8 weeks of age weighing between 20 g and 25 g were randomly divided into two groups of 10 per group. The animals were fed in a standard room with controlled temperature and humidity and were fed a regular diet. The mice were placed under general anesthesia via intraperitoneal injection of 1% sodium pentobarbital. Experimental periodontitis was induced by gentle placement of sterilized 5-0 cotton ligatures around the bilateral maxillary and mandibular second molars, followed by mesiobuccal knotting. In mice with ligature-induced experimental periodontitis, the ligatures were maintained in position throughout the 10-day experiment with no need for replacement. Non-ligatured mice were used as negative controls. All experiments were performed according to the approved institutional guidelines of the Peking University Biomedical Ethics Committee.

2.7 | Analyses of mice periodontal tissue destruction in vivo

Ten days after ligature placement, the jaw was surgically removed and fixed in 4% paraformaldehyde. To assess the bone loss, the specimens were scanned with high-resolution Inveon micro-computed tomography (μCT; Siemens) following the experimental settings: 80 kV X-ray voltage, 500 μA node current, and 1500-ms exposure time for each of the 360 rotational steps. Then, three-dimensional (3D) images were reconstructed with multimodal 3D visualization software. Thereafter, the samples were decalcified in 10% EDTA (pH 7.2 ± 0.2) at 37°C for two weeks with three solution changes made per week. Serial paraffin sections of 5 μm in thickness were obtained from the buccal-lingual aspects of the second molars and were stained with H&E for histological evaluation. The locations of IL-6 in the periodontal tissues were detected by immunocytochemistry using a rabbit monoclonal antibody to IL-6 (Beijing Biosynthesis Biotechnology) in accordance with the manufacturer's instructions. Images were captured using a light microscope (BX51/DP72; Olympus). IRS with modification was used to evaluate immunohistochemical staining density and statistical analyses.

2.8 | Complete blood cell analyses of mice with experimental periodontitis and controls

Blood was collected in heparinized tubes (Greiner Bio-One) by cardiac puncture. A technician who was blinded to the experimental grouping performed complete blood cell analyses of the samples using a calibrated automated hematology analyzer (Sysmex).

2.9 | Measurement of the concentrations of IL-6 and hepcidin in blood samples

The serum was separated by centrifugation and was immediately stored at −80°C. The concentrations of IL-6 and hepcidin were measured using ELISA kits (Gersion; Meinian Industrial, respectively) according to the manufacturer's instructions. All samples were measured in triplicate.

2.10 | Hepatocyte culture and stimulation

Human liver cell line L-02 and mouse liver cell line NCTC 1469 were obtained from the Type Culture Collection of the Chinese Academy of Sciences. The L-02 cells and NCTC-1469 cells were cultured in Gibco Dulbecco's modified Eagle medium (DMEM; Gibco) and Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco), respectively, both of which contained 10% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin (Gibco). All cultures were maintained at 37°C under 5% CO_2. Human liver cells and mouse liver cells were treated with and without 20 ng/ml and 100 ng/ml IL-6 (PeproTech) for 8 h and 24 h. Afterward, the cells and culture supernatants were collected for mRNA examination and protein secretion.

2.11 | RNA extraction and real-time quantitative polymerase chain reaction (RT-qPCR)

The total RNA from the hepatocytes and liver was extracted using TRIzol (Invitrogen)-based methods. The RNA was reverse

transcribed to cDNA using a Reverse Transcription System (Toyobo). Subsequently, RT-qPCR was performed using PowerUp™ SYBR™ Green Master Mix (Roche) according to the manufacturer's protocol. The primer sequences for the target genes were designed as described in Table S1. All reactions were carried out in triplicate. The relative mRNA expression in each sample was then normalized to *β-actin* (mouse) or *GAPDH* (human) expression using the $2^{-\Delta\Delta Ct}$ method.

2.12 | Measurement of hepcidin secretion concentrations by ELISA

The culture supernatant was collected, centrifuged, and stored at −80°C. The concentrations of hepcidin by human and mouse liver cells were measured using ELISA kits (R&D Systems; Meinian Industrial, respectively) according to the manufacturer's instructions.

2.13 | Statistical analysis

Variables were presented as mean ± standard deviation (SD, normal distribution). Clinical and blood parameters were compared between the AgP group and the control group. Statistical analyses were conducted using Student's *t* tests and Mann–Whitney tests for normal and abnormal distribution, respectively, and the *chi-square* test for categorical variables. The associations between AgP and blood parameters were determined by generalized estimate equation and logistic regression modeling adjusted for age, gender, and body mass index (BMI). The results of cell experiments were acquired at least in triplicate in three separate experiments, and the differences among groups were analyzed using one-way ANOVA. All statistical analyses were performed with SPSS 20.0 (SPSS Inc.) software. A two-tailed *p*-value below .05 was considered statistically significant.

3 | RESULTS

3.1 | Demographic features and periodontal status of the subjects

In total, 98 AgP patients and 103 healthy subjects were enrolled in this study; these groups included 21 (21.43%) and 37 (35.92%) males, respectively, having mean ages of 26.23 ± 5.08 and 30.43 ± 10.17, respectively. The BMI in the AgP group, at 18.79 ± 1.32 kg/m^2, was lower than that in the control group, at 21.47 ± 2.15 kg/m^2 ($p < .001$). For clinical periodontal parameters in the AgP group, the mean values of PD, BI, and AL were 4.82 ± 1.04 mm, 3.53 ± 0.52, and 4.52 ± 1.82 mm, respectively. All were statistically significantly higher than the control group ($p < .001$) (Table 1).

3.2 | Local periodontal inflammation and systemic inflammation in the AgP group

The H&E staining revealed little inflammatory cell infiltration in the healthy gingival tissues (Figure 1A), whereas extensive inflammation was present in tissues from AgP patients (Figure 1B). Moreover, vasodilation, vascular proliferation, and dense leukocyte infiltration were present in the connective tissues of inflammatory gingiva from patients with AgP (Figure 1B). Further, immunohistochemical staining was performed on the inflammatory gingival samples. In contrast to that in healthy gingival tissues (Figure 1C), the IL-6 levels were up-regulated in the areas of inflammatory cell infiltration in the inflamed gingiva from patients with AgP ($p = .001$; Table S2) (Figure 1D). The levels of systemic inflammatory markers, including WBC counts (6.43 ± 1.91 × 10^9/L vs. 5.62 ± 1.19 × 10^9/L, $p = .004$), IL-6 (1.81 ± 0.19 ng/L vs. 0.61 ± 0.93 ng/L, $p < .001$), and CRP (3486.14 ± 497.74 μg/L vs. 1218.12 ± 243.78 μg/L, $p < .001$), were significantly higher in the AgP group (Table 1).

3.3 | Changes in erythrocyte parameters manifested in the AgP group and the phenomenon of AI

The mean HGB, HCT, and MCV were significantly lower in the AgP group than those in the control group (136.96 ± 13.41 g/L vs. 141.16 ± 14.32 g/L, 0.40 ± 0.04 L/L vs. 0.41 ± 0.04 L/L, and 88.02 ± 4.89 fl vs. 89.47 ± 4.64 fl, respectively; $p < .05$). The RBC counts and MCH levels were also relatively lower in AgP patients, although the differences did not reach significance (Table 1). When the data were divided into male and female patients, the lower levels of HGB and HCT had no statistical difference (Table S3). The adjusted linear regression analyses after adjusting for age, gender, and BMI revealed statistically significant correlations between AgP and decreased HGB ($\beta = -4.20$, 95% confidence interval (CI): −0.36, −8.04, $p = .033$), HCT (adjusted $\beta = -0.01$, 95% CI: 0.00, −0.02, $p = .030$), and MCV (adjusted $\beta = -2.04$, 95% CI: −0.38, −3.71, $p = .017$) levels (Table 2). According to the World Health Organization (WHO) definition for anemia (HGB <120 g/L in women and <130 g/L in men),[20] 4.08% of AgP patients were anemic (Table 3). The frequencies of subjects with HCT below the laboratory reference values in AgP patients were statistically significantly higher than those in the healthy group ($p < .05$).

3.4 | Periodontitis in mice and up-regulated expression of IL-6 in periodontal tissues induced by ligature placement

Advanced lesions formed after 10 days of continued plaque accumulation. The μCT images showed severe alveolar bone loss in the mice with ligature (Figure 2A). Histologic analyses showed compared with

TABLE 1 Parameters of the study subjects

Variables	Healthy group	AgP group	*p*-value
Demographic features			
Number	103	98	
Age	30.43 ± 10.17	26.23 ± 5.08	<.001*
Gender (M/F)	37/66	21/77	.023*
BMI (kg/m^2)	21.47 ± 2.15	18.79 ± 1.32	<.001*
Periodontal clinical parameters			
PD (mm)	1.68 ± 0.43	4.82 ± 1.04	<.001*
BI	1.20 ± 0.25	3.53 ± 0.52	<.001*
AL (mm)	0.00 ± 0.00	4.52 ± 1.82	<.001*
Inflammatory markers			
WBC (10^9/L)	5.62 ± 1.19	6.43 ± 1.91	.004*
IL-6 (ng/L)	0.61 ± 0.13	1.81 ± 0.19	<.001*
CRP (μg/L)	1218.12 ± 243.78	3486.14 ± 497.74	<.001*
Anemia-related indicators			
RBC (10^{12}/L)	4.59 ± 0.48	4.50 ± 0.40	.168
HGB (g/L)	141.16 ± 14.32	136.96 ± 13.41	.033*
HCT (L/L)	0.41 ± 0.04	0.40 ± 0.04	.011*
MCV (fl)	89.47 ± 4.64	88.02 ± 4.89	.032*
MCH (pg/cell)	30.86 ± 2.13	30.48 ± 2.28	.222
MCHC (g/dl)	344.81 ± 14.01	346.04 ± 14.39	.538

Note: Data are presented as mean ± SD/N. Between-group comparisons were performed using *t* test, chi-square test or Mann–Whitney *U* test.

Abbreviations: AgP, Aggressive periodontitis; BMI, body mass index; PD, probing depth; BI, bleeding index; AL, attachment loss; WBC, white blood cell; IL-6, interleukin-6; CRP, C-reactive protein; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration.

*$p < .05$.

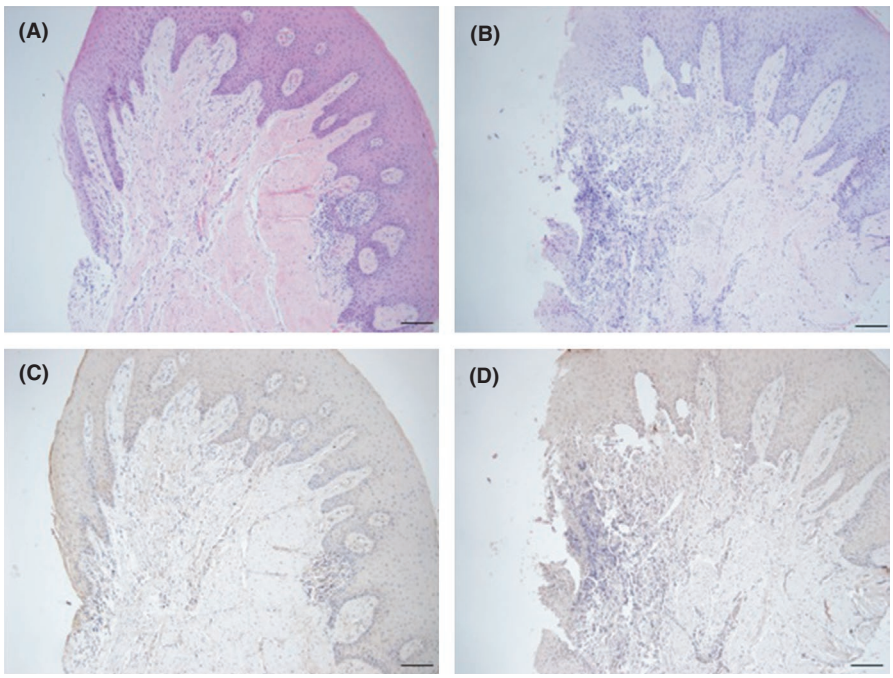

FIGURE 1 Histological and immunohistochemical appearances of gingival tissue samples from periodontitis patients and healthy subjects. (A) H&E staining of healthy gingival tissue. (B) H&E staining of gingival tissue from periodontitis patients. (C) IL-6 expression in healthy gingival tissue. (D) Elevated expression of IL-6 in gingival tissue from periodontitis patients. Magnification, 10×; scale bar: 100 μm

TABLE 2 Comparison of blood parameters between AgP patients and the healthy group

		No adjusted		Adjusted	
Variables	**Group**	**β 95% CI**	***p*-value**	**β 95% CI**	***p*-value**
	Healthy	Reference		Reference	
WBC (10^9/L)	AgP	0.81 (0.32, 1.30)	.002*	0.94 (0.32, 1.57)	.003*
RBC (10^{12}/L)	AgP	−0.09 (−0.21, 0.04)	.168	−0.02 (−0.14, 0.10)	.792
HGB (g/L)	AgP	−4.20 (−8.04, −0.36)	.033*	−1.45 (−5.50, 2.61)	.485
HCT (L/L)	AgP	−0.01 (−0.02, −0.00)	.011*	−0.01 (−0.02, 0.00)	.092
MCV (fl)	AgP	−1.45 (−2.77, −0.13)	.032*	−2.04 (−3.71, −0.38)	.017*
MCH (pg/cell)	AgP	−0.38 (−0.99, 0.23)	.222	−0.29 (−1.08, 0.50)	.477
MCHC (g/dl)	AgP	1.23 (−2.69, 5.16)	.538	4.75 (−0.31, 9.81)	.067

Note: The comparison of blood parameters between AgP patients and the healthy group was analyzed by generalized estimate equation and linear regression modeling.
Adjusted for age, gender, and BMI.
*$p < 0.05$.

the results of the control group (Figure 2B), significant alterations in the periodontal tissues contained dilated capillaries, dense inflammatory cell infiltration, and collagen breakdown. The epithelial-connective tissue interfaces and large areas of collagen-depleted connective tissues showed infiltration of numerous inflammatory cells along with intercellular edema (Figure 2C). The histological analyses of periodontal tissues following ligature application revealed infiltration of inflammatory cells with up-regulated levels of IL-6 appeared in the connective tissues of the experimental periodontitis mice (Figure 2E) compared with the control group (Figure 2D) ($p < .001$; Table S2).

3.5 | Systemic inflammation in mice caused by ligature-induced experimental periodontitis

The WBC counts in the experimental periodontitis group were significantly higher than those in the control group ($p < .05$) (Figure 3A). In mice with ligature-induced experimental periodontitis, higher levels of pro-inflammatory cytokine IL-6 were detected compared with that in the control mice ($p < .05$) (Figure 3B).

3.6 | Phenomenon of AI detected in mice with experimental periodontitis

The RBC counts, HGB, HCT, and MCV values ($7.91 \pm 0.37 \times 10^{12}$/L vs. $9.65 \pm 0.87 \times 10^{12}$/L, 124.00 ± 4.00 g/L vs. 151.33 ± 12.06 g/L, 0.39 ± 0.01 L/L vs. 0.47 ± 0.04 L/L, and 48.16 ± 1.12 fl vs. 49.50 ± 0.45 fl, respectively; $p < .05$) were reduced in the mice with ligature-induced experimental periodontitis (Figure 4A–D). Significantly higher coefficients of variation of red blood cell distribution width (RDW-CV) and red blood cell volume distribution width standard deviation (RDW-SD) were also observed in these mice (Figure 4E,F).

3.7 | Gene expression of hepcidin and serum hepcidin levels increased by ligature-induced experimental periodontitis

The RT-qPCR assays revealed a fivefold increase in hepcidin mRNA levels in the livers of mice with ligature-induced experimental periodontitis ($p < .01$) (Figure 5A). Moreover, higher serum levels of hepcidin were measured in these mice (19.15 μg/L vs. 15.62 μg/L, $p < .01$) (Figure 5B).

3.8 | Hepcidin expression in both human and mouse liver cells induced by IL-6

After IL-6 stimulation, the expression of hepcidin mRNA and hepcidin secretion levels was both up-regulated at 8 h and 24 h compared with that in the control group. IL-6 treatment induced approximately fourfold hepcidin expression compared with that in the untreated

TABLE 3 Number (%) of control subjects and AgP patients with values for erythrocyte parameters outside the reference ranges

Variables	**Healthy group (%)**	**AgP group (%)**	***p*-value**
RBC outside ref.	1 (0.97)	2 (2.04)	.532
HGB outside ref.	2 (1.94)	4 (4.08)	.373
HCT outside ref.	11 (10.68)	21 (21.43)	.037*
MCV outside ref.	3 (2.91)	3 (3.06)	.951
MCH outside ref.	3 (2.91)	5 (5.10)	.427
MCHC outside ref.	12 (11.65)	20 (20.41)	.090

Note: Between-group comparisons were performed using the chi-square test.
The reference ranges are as follows: RBC, male: $4.0–5.50 \times 10^{12}$/L, female: $3.5–5.0 \times 10^{12}$/L; HGB, male: 120–160 g/L, female: 110–150 g/L; HCT, male: 0.40–0.50 L/L, female: 0.37–0.48 L/L; MCV, 80–94 fl; MCH, 26.0–34.0 pg/cell; MCHC: 320–360 g/dl.
*$p < .05$.

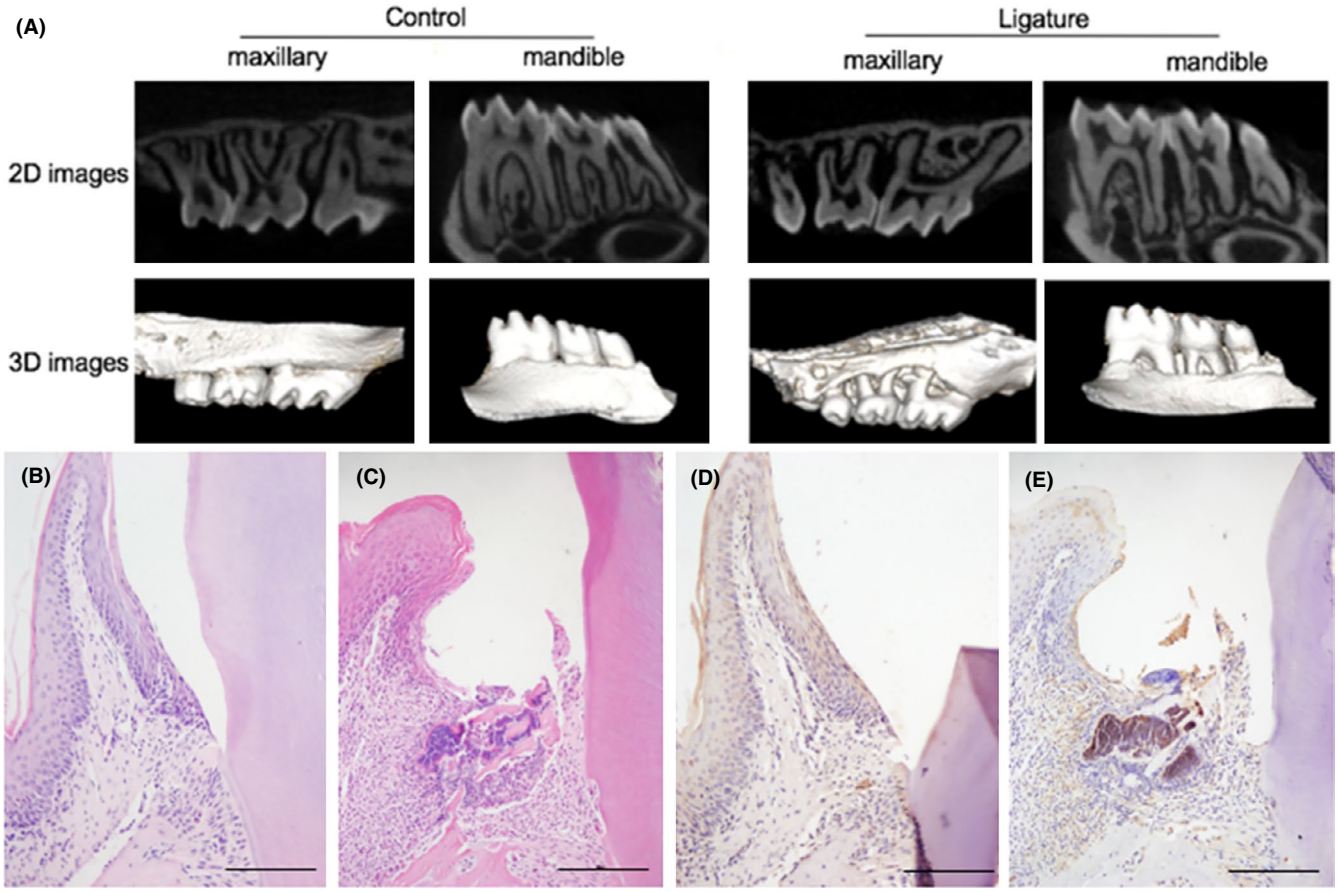

FIGURE 2 μCT images and histological appearances of murine periodontal tissues. (A) Two-dimensional and three-dimensional μCT images of the maxillary and mandible molars of different animals from each group at day 10. (B) H&E staining of periodontal tissues in the control group. (C) Bone resorption and intense inflammatory infiltration in periodontal tissue in mice with experimental periodontitis. (D) IL-6 expression in periodontal tissues of control mice. (E) Elevated expression of IL-6 in periodontal tissues of mice with ligature-induced periodontitis. Magnification, 10×; scale bar: 100 μm

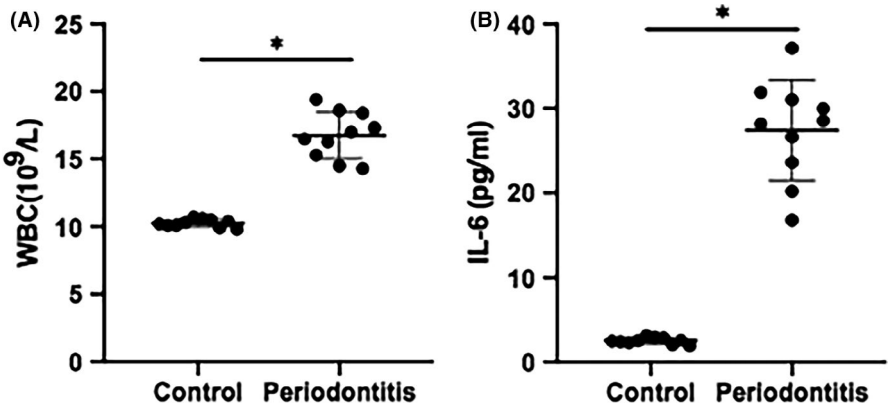

FIGURE 3 (A) Mean WBC counts and (B) serum IL-6 concentrations of mice in the control group (n = 10) and those in the ligature-induced periodontitis group (n = 10). Data are presented as mean ± SD. Between-group comparisons were performed using Student's t test or the Mann–Whitney U test; *p < .05 as compared with the control group

control in human liver cells (Figure 6A). The hepcidin concentration in the supernatant was also higher in the IL-6 treatment groups (Figure 6B). Similarly, higher hepcidin expression levels were found in the mouse liver cells (Figure 6C,D). The treatment of hepatocytes with 100 ng/ml IL-6 increased the hepcidin mRNA up to ninefold within 24 h.

4 | DISCUSSION

In the present study, we observed lower HGB and HCT levels among AgP patients. Regression analyses after adjusting for age, gender, and BMI still revealed significant correlations between AgP and decreased HGB, HCT, and MCV values. These results

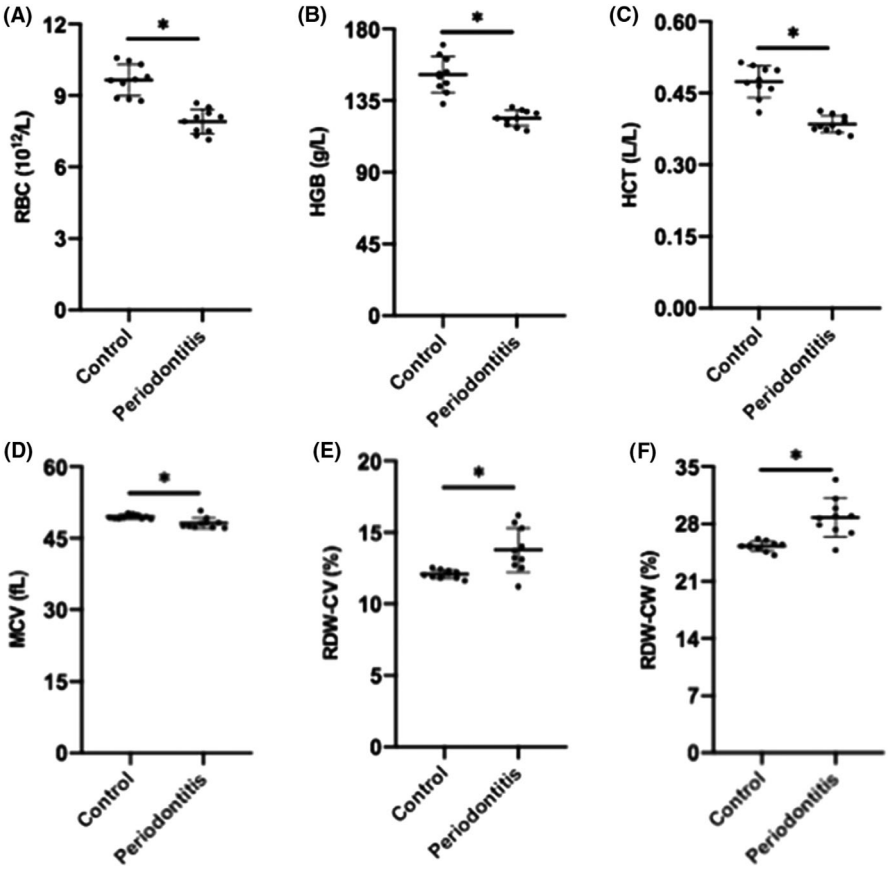

FIGURE 4 (A) Mean RBC counts, (B) HGB, (C) HCT, (D) MCV, (E) RDM-CV, and (F) RDW-SD of mice in the control group (n = 10) and in the ligature-induced periodontitis group (n = 10). Between-group comparisons were performed using Student's t test or the Mann–Whitney U test; *p < .05 as compared with the control group

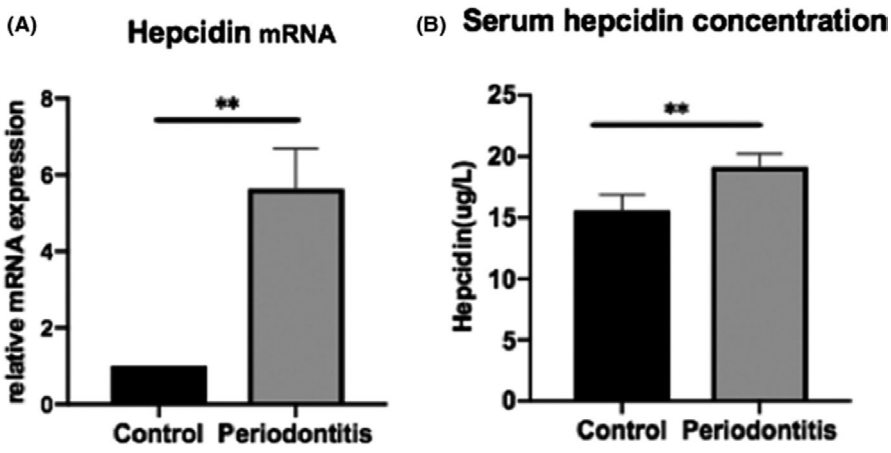

FIGURE 5 (A) Relative mRNA expressions of hepcidin gene and (B) serum hepcidin concentrations in livers of ligature-induced periodontitis mice (n = 10); *p < .05 and **p < .01 as compared with the control group (n = 10). All reactions were carried out in triplicate

indicate that AgP can activate systemic immune-inflammatory responses and eventually increase the risk of AI. The healthy subjects were recruited with periodontal health and systemic health. Current or previous smokers were excluded because smoking is considered as a cofactor for the development of both periodontitis[21] and anemia.[22] Tendencies toward periodontitis-related AI have also been observed in other clinical researches[16] and meta-analyses.[17,18] A recent systematic review and meta-analysis enrolled 1423 periodontitis patients demonstrated a lower level of HGB, RBC, and MCV, and this pooled result confirmed the association between periodontitis and the tendency of AI.[18] Interestingly, a recent study with a sample size of 125 AgP patients and 121 CP patients reported no differences in leukocyte and erythrocyte parameters between the two groups.[23] Although the diversity of different studies remained, they have all reported the development tendency of AI in periodontitis patients. Possible explanations for these differences were as follows: (a) different sample size in different studies; (b) racial heterogeneity of erythrocyte parameters; (c) different age and male/female ratio of enrolled subjects; (d) different criteria of periodontitis patients and healthy controls; (e) there might be additional confounders that not accounted for in the observed relationship between periodontitis and AI. Our findings, coupled with those of previously published studies,[16–18,23,24] suggest that both CP and AgP can increase the risk of AI as well as that of other inflammation-related diseases.

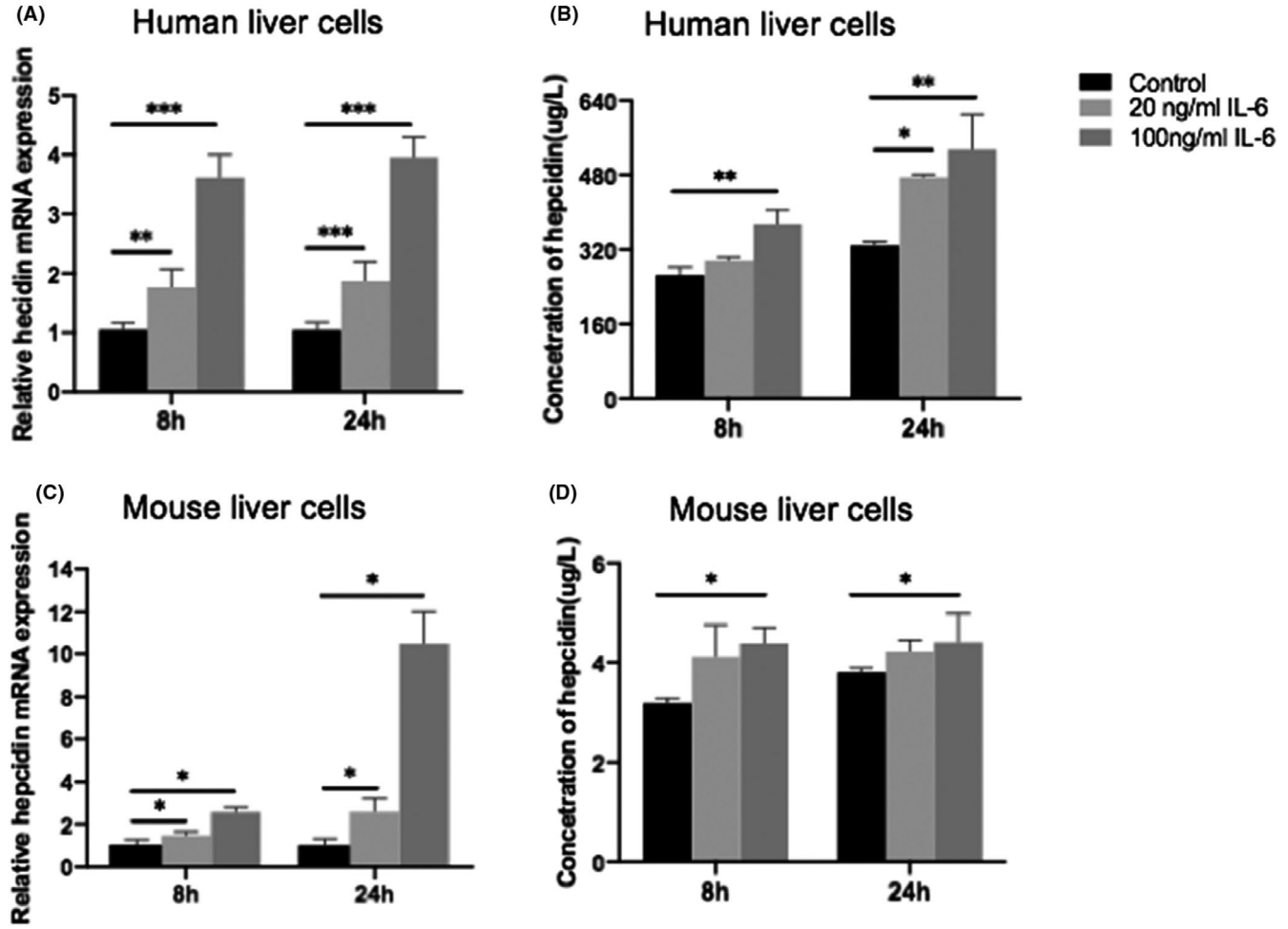

FIGURE 6 (A) Relative mRNA expressions of hepcidin gene and (B) supernatant hepcidin concentrations in human liver cells; those in mouse liver cells are presented in (C) and (D), respectively; $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$ as compared with the control group. Results were acquired at least in triplicate in three separate experiments

However, the changes in HGB and HCT values in our study were not as striking as those observed in anemia caused by other inflammatory conditions, such as rheumatoid arthritis,[25] neoplastic conditions,[26] and inflammatory bowel disease.[27] AI is observed within 33%–60% of patients with rheumatoid arthritis[25] and occurred in 11.1% at hemoglobin <110 g/L.[28] 41% of cancer patients were anemic, and the majority of them developed mild-to-moderate anemia (hemoglobin 100 to 119 g/L).[26] In our study, the prevalence of periodontitis-related anemia was 4.08%, which was more mild compared other inflammatory diseases. This difference can be explained by the fact that periodontitis is a rather chronic and milder inflammatory condition compared with other systemic infections or conditions. Further studies are required to correlate different severities of periodontal inflammation and its effects on systemic conditions.

In the present study, ligature-induced experimental periodontitis in mice caused significant infiltration of inflammatory cells in periodontal tissues. The hematological analyses of erythrocyte parameters showed dramatic decreases in RBC counts as well as lower HGB and HCT values in mice with ligature-induced experimental periodontitis, which were manifested as anemia.[29] The apparent increase in WBC counts and IL-6 levels confirmed systemic inflammation by periodontitis. These observations demonstrate that the development of anemia in experimental periodontitis is attributed to periodontal infection. The accumulation of periodontal pathogens led to inflammatory cascade reaction that was continued by the local inflammatory reaction and subsequently by an overall higher degree of systemic inflammation. A strong relationship was noted between periodontitis and AI, in which immuno-inflammatory responses caused by periodontal inflammation led to an increased risk for AI.

Until recently, little was understood about the pathogenesis of periodontitis-related AI; therefore, the mechanisms underlying this condition were further explored. It now appears that pro-inflammatory cytokine IL-6 induces the production of hepcidin, the iron-regulatory hormone, and the key mediator of AI[11–13] that could be responsible for erythrocyte parameter changes in periodontitis-related AI. Using mice as experimental periodontitis models, our findings highlight the principle function of hepcidin as the central pathologic mediator and imply the importance of the IL-6-hepcidin

axis in the development of AI in periodontitis patients. Hepcidin, a 25-amino acid peptide iron-regulatory hormone secreted by the liver, is known as the key mediator of AI.[30,31] Hepcidin is also regarded as an acute-phase response peptide and mediator of innate immunity during inflammatory status.[32] We speculate that hepcidin is important in the pathogenesis of periodontitis-related AI. Hepcidin functions by blocking the main iron flows into plasma and is up-regulated by inflammation.[33] Increased circulation of hepcidin owing to inflammatory stimulation binds to ferroportin, the only known cellular iron exporter found on the cell surfaces, which induces its internalization and degradation and inhibits macrophage iron release and intestinal iron absorption.[34] The limited availability of iron leads to decreased iron delivery for erythropoiesis and eventually the development of anemia.[35] The impairment of iron delivery by macrophages and enterocytes is also thought to be part of the host defense mechanism of fighting infection by restricting the availability of extracellular iron as a nutrient for the invading microbes.[36] Because hepcidin is a small, evolutionarily conserved peptide, it is difficult to generate antibodies for laboratory assays.[37] Immunoassay currently regarded as the most effective measurement method.[38] Therefore, we used RT-qPCR and ELISA for hepcidin quantitative analyses.

One key finding of the present study is that ligature-induced experimental periodontitis in mice leads to a greater than fivefold induction of hepcidin and higher serum hepcidin concentrations. Similar findings have been reported in an inflammation model of turpentine injection that showed 6-fold[39] and a 12-fold increases[40] in hepcidin mRNA levels. Other studies revealed that serum hepcidin levels in chronic periodontitis groups were higher and suggested that the absence or presence of chronic periodontitis was a significant predictor of increased serum hepcidin concentrations.[41,42] Moreover, after nonsurgical periodontal therapy, chronic periodontitis patients showed decreased levels of prohepcidin, the prohormone of hepcidin, suggesting that the inflammatory burden improved after treatment.[43] These phenomena are largely consistent with the conclusion that increased hepcidin concentrations in serum are pathogenic in periodontitis-related AI.[35] Thus, our findings demonstrate that hepcidin plays a pivotal mediator role in the development of concomitant periodontitis and AI.

The important link between inflammatory cytokines and hepcidin has been verified in the pathogenesis of AI.[44] One common pathophysiological mechanism underlying AI is that the invasion of microorganisms leads to immune cell activation and the release of numerous cytokines.[32] Among these cytokines, the IL-6-hepcidin axis appears to be the most important in the development of AI.[45] In vitro stimulation of human hepatocytes with a panel of cytokines showed strong induction of hepcidin mRNA by IL-6, but not interleukin-α (IL-α) or tumor necrosis factor-α (TNF-α); this response could be ablated by the addition of anti-IL-6 antibodies.[40] In vivo, the induction of hypoferremia by IL-6 and hepcidin occurred within a few hours and was not observed in IL-6-knockout mice treated with turpentine as a model of inflammation.[46,47] Thus, we attempted to explore whether IL-6-stimulated hepcidin is involved in the underlying mechanisms of periodontitis-related AI in the mouse model of experimental periodontitis. In the ligature-induced periodontitis model, histological analyses showed infiltration of inflammatory cells with up-regulated IL-6 in the gingival tissue. Moreover, the ELISA assay revealed higher levels of IL-6. Apparently, the increase in WBC counts and the elevated levels of IL-6 in the experimental periodontitis mice are consistent with the inflammatory state in patients with periodontitis. Numerous studies have concluded that IL-6 was up-regulated both in periodontitis patient[5,48] and experimental periodontitis models.[49,50] These data provide strong support for the speculation that pro-inflammatory cytokine IL-6 is a primary inducer of increased hepcidin and that the IL-6-hepcidin axis might be necessary for the development of periodontitis-associated AI.

To further confirm the importance of the IL-6-hepcidin regulatory pathway, we treated human and mouse liver cells with IL-6. Similar to that reported in previous research,[40] the treatment of hepatocytes with IL-6 increased both hepcidin mRNA expression and hepcidin secretion. Pro-inflammatory cytokine IL-6-induced hepcidin might also be a central mediator in the development of AI in periodontitis cases. These data together with our results confirm that periodontal inflammation leads to systemic immune activation and dramatically elevated IL-6 levels, which act on hepatocytes to induce hepcidin production and eventually increase the risk of AI. Previous research indicates that hepcidin expression is inflammation-dependent, mediated through IL-6/JAK/STAT3 signaling, and iron-dependent, controlled through BMP/SMAD signaling.[51] Inflammatory signaling, initiated by the release of IL-6, activates the JAK/STAT pathway, resulting in STAT3 phosphorylation, which then binds the hepcidin promoter and increases hepcidin expression.[52] BMP6 signaling involves activation and phosphorylation of the BMP receptors, followed by phosphorylation of SMAD1/5/8 and the translocation of the complex into the nucleus, where it binds to a response element in the hepcidin promoter.[53] Future research will be conducted to determine whether this signal pathway is involved in periodontitis-induced AI. Apart from inflammation-induced cytokines and increased hepcidin as the major regulators of AI, shortened erythrocyte survival and suppressed erythropoietin response to anemia have been reported as minor factors contributing to AI in a disease-specific pattern.[54] Further studies are needed to explore whether these mechanisms participate in periodontitis-related AI.

The changes in blood parameters related to anemia such as fewer RBC numbers and lower levels of HGB and HCT suggest concomitant periodontitis and AI. Periodontitis should be considered as another chronic inflammatory disease that can contribute to AI. These subtle changes should not be ignored. Although they may not be cause for concern in healthy individuals, these factors can contribute to the inflammatory burden and disorders in erythropoiesis when associated with other system inflammatory conditions. Blood parameter analyses including inflammatory markers and anemia-related indicators are necessary in clinical periodontal practice. Hepcidin can be used as a biomarker to predict the risk of AI in periodontitis patients. Furthermore, to our knowledge, this is the first study to report that hepcidin together with the IL-6-hepcidin axis plays a central role in

the development of periodontitis-related AI. However, additional studies are necessary for explaining the function of hepcidin and other meticulous mechanisms in the pathogenesis of periodontitis-related anemia. In addition, future research will focus on whether periodontal therapy can prevent or minimize the risk of AI.

5 | CONCLUSIONS

In this study, we analyzed the correlations between periodontitis and lower HGB, HCT, and MCV values. Our findings suggested that immuno-inflammatory responses by periodontal inflammation lead to increased risk for AI. Pro-inflammatory cytokine IL-6-induced hepcidin is suggested to play a central mediator role and act as a key pathologic mechanism. Further, periodontitis may be considered as an additional inflammatory disease that contributes to the development of AI.

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CONFLICT OF INTEREST

The authors declare that there are no competing interests respect to the authorship and/or publication of this article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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