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Hyperhomocysteinaemia aggravates periodontitis by suppressing the Nrf2/HO-1 signalling pathway

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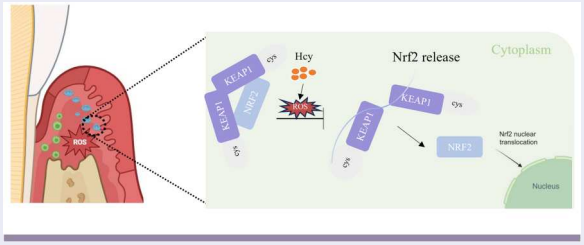
ABSTRACT

Periodontitis, a common dental illness, causes periodontal tissue inflammation and irreversible bone loss, inevitably resulting in tooth loss. Hyperhomocysteinaemia (HHcy), defined as blood total homocysteine (Hcy) levels greater than 15 μmol/L, is linked to increased cardiovascular disease risk. Mounting evidence indicates a connection between HHcy and periodontitis; however, the underlying processes remain unknown. Herein, we explored the mechanisms by which HHcy exacerbates periodontal tissue inflammation and osteoclast formation. In an animal model of periodontitis treated with HHcy, periodontal attachment loss was aggravated, and both systemic and gingival tissue inflammation levels tended to increase; additionally, antioxidant-related proteins were suppressed and expressed at low levels, whereas oxidative damage-related protein expression increased. In RAW264.7 cells treated with LPS or LPS + Hcy, the LPS + Hcy group presented increased reactive oxygen species (ROS) fluorescence intensity, and Nrf2/HO-1 signalling pathway suppression was associated with inflammatory cytokine (TNF-α) expression. In monocyte osteoclasts treated with Rankl or Rankl + Hcy, the Rankl + Hcy group presented Nrf2/HO-1 signalling pathway suppression, an increase in osteoclast-related proteins (NFATc-1 and CTSK), and a more pronounced osteoclastic phenotype. Therefore, HHcy may exacerbate inflammation severity and osteoclast generation in periodontitis by promoting ROS production and inhibiting the Nrf2/HO-1 signalling pathway.



KEYWORDS

Periodontitis; oxidative stress; ROS; Nrf2; bone loss; inflammation; innate immunity; hyperhomocysteinaemia

1. Introduction

Periodontitis (PD) mainly arises from a disruption in the equilibrium of the oral microbial community, triggering localized inflammatory reactions and initially activating the innate immune system [1]. Macrophage-mediated inflammation serves as the first line of defence against periodontal infections but can also lead to the destruction of periodontal tissues. This ecological imbalance gradually destroys the tooth-supporting structures, leading to tooth loosening and eventually tooth loss [2–4]. Homocysteine (Hcy) is an intermediate metabolite of methionine and is widely involved in biological homeostasis and disease mechanisms within the body, including promoting cellular autophagy, apoptosis, and oxidative stress. Hyperhomocysteinaemia (HHcy) is characterized by serum total homocysteine (Hcy) concentrations greater than 15 μmol/L [5]. Previous studies have shown that people with periodontitis have significantly elevated Hcy concentrations in both their gingival crevicular fluid and blood serum. Increased Hcy concentrations are correlated directly with clinical periodontal parameters such as the gingival index (GI), plaque index (PI), bleeding on probing (BOP), probing depth (PD), and clinical attachment level (CAL) [6–8]. Moreover, after patients received

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folic acid supplementation to reduce Hcy levels, the aforementioned indices improved in patients with periodontitis [9]. These findings imply that HHcy could be a significant contributor to the progression of PD; however, the precise pathogenic mechanisms remain to be elucidated.

HHcy is recognized as a contributing factor to an array of systemic conditions, including atherosclerosis (AS), cardiovascular disease (CVD), type 2 diabetes, and Alzheimer's disease [10, 11]. HHcy can damage the cells lining blood vessels by triggering an inflammatory response and the accumulation of reactive oxygen species (ROS), in conjunction with the development of oxidative stress and cellular hypomethylation, causing damage to the vascular intima and thus exacerbating the progression of AS and CVD. Hcy induces ROS accumulation by upregulating NADPH oxidase expression in endothelial cells and suppressing the antioxidant pathway mediated by glutathione peroxidase (GPX) [12]. Simultaneously, oxidative stress, endoplasmic reticulum stress, inflammatory responses, and epigenetic alterations might constitute the pathways through which HHcy intensifies Alzheimer's disease [13].

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a crucial transcription factor that shields organisms from oxidative damage [14]. A deficiency in or the suppression of Nrf2 can result in the worsening of numerous inflammatory diseases, including Hyperhomocysteinaemia, sepsis, pleurisy, and emphysema [15,16]. Nrf2 may promote the production of antioxidant-related elements via redox-sensitive signalling pathways, providing cells with protection against various types of oxidative damage. In general, Nrf2 is confined to the cytoplasm and interacts with Kelch-like ECH-associated protein 1 (KEAP1). This interaction causes Nrf2 degradation via the CUL3 ubiquitin protease system. ROS cause Nrf2 to be expelled from the KEAP1-Nrf2 complex under oxidative stress, allowing Nrf2 to be transported to the nucleus. This gene affects the transcription of genes driven by antioxidant response elements (AREs) and a variety of detoxifying enzymes, including glutathione synthetase (GSH), quinone oxidoreductase 1 (NQO1), haem oxygenase-1 (HO-1), superoxide dismutase (SOD), and glutathione peroxidase (GPX) [17]. These proteins have the ability to lessen the harm that oxidative stress causes to proteins, lipids, and DNA [18]. Similarly, Nrf2 plays a protective role in periodontitis, primarily by countering oxidative stress, curbing the activation of osteoclasts, and regulating the growth, maturation, and death of periodontal cells [19].

It is still uncertain whether HHcy exacerbates periodontitis by increasing oxidative stress, and the related mechanisms involved are unknown. In this study, we investigated the role of oxidative stress in periodontitis in combination with HHcy, as well as the role of HHcy in intensifying the inflammatory response of macrophages and the differentiation of osteoclasts, and explored the potential regulatory mechanisms involved. Our research results suggest that HHcy may promote the inflammatory response of macrophages and the differentiation of osteoclasts in periodontal tissues by inhibiting the Nrf2/HO-1 signalling pathway, thereby exacerbating the onset and progression of periodontitis.

2. Materials and methods

2.1. Animals and group allocation

Six-week-old male C57BL/6J mice were procured from GemPharmatech Co., Ltd., China. The mice were maintained in a specific pathogen-free environment with regulated temperature (ranging from 22 to 24 degrees Celsius) and humidity levels under a 12-hour alternating light/dark cycle. Twenty-four mice were randomly divided into four groups, with six mice per group. All protocols used in this research were approved by the Laboratory Animal Management and Use Committee at Nanchang University (Ethics Approval Number: NCULAE-20221031158).

To establish HHcy in the experimental model, the mice were provided with a diet rich in protein (containing 25% casein) for a period of two weeks. Blood was then collected from the tail vein to measure homocysteine (Hcy) levels [20]. Successful modelling was indicated when the serum Hcy levels in the mice exceeded 15 μmol/L. After general anaesthesia with isoflurane inhalation, experimental periodontitis was induced in mice by the placement of a ligature around the neck area of the maxillary bilateral second molars using sterile 5–0 silk sutures [21].

The animal experimental groups were as follows: (1) control group, normal diet; (2) hyperhomocysteinaemia group (HHcy), high-protein diet (25% casein); (3) periodontitis group (PD), ligature placement around the maxillary second molars; and (4) periodontitis + hyperhomocysteinaemia group (PD + HHcy), high-protein diet (25% casein) + ligature placement around the maxillary second molars.

Following a four-week period, the mice were humanely sacrificed. Throughout the study, body weight and ligature condition were monitored on a weekly basis; any mice that died were not included in the final analysis. Mouse serum was collected for ELISAs, and gingival tissues were analysed for gene expression. Maxillae were immersed in a 4% paraformaldehyde solution for 24 hours prior to subsequent analyses.

2.2. Microcomputed tomography analysis

To assess the loss of alveolar bone attachment, high-resolution microcomputed tomography (micro-CT; VivaCT40, SCANCO Medical AG, Switzerland) with a resolution of 12.5 μm was utilized. Three-dimensional reconstructions were performed using Mimics software (Materialise, V21.0). The degree of attachment loss was determined by calculating the average distance from the cementoenamel junction (CEJ) to the alveolar bone crest of the maxillary second molar. This measurement was taken on the sagittal plane, considering the palatal aspect at three points: mesially, centrally, and distally.

2.3. Histological analysis

Mouse gingival tissues and maxillae were preserved in a 4% paraformaldehyde solution, and maxillae were decalcified. The samples were subsequently dehydrated, and 7-μm-thick frozen sections were prepared. Sections were stained with haematoxylin and eosin (H&E) using a staining kit (Servicebio, Wuhan, China) following the protocol provided by the manufacturer. Osteoclasts were stained using a tartrate-resistant acid phosphatase (TRAP) staining kit (Servicebio, Wuhan, China). The stained sections were then examined, and images of both H&E and TRAP staining were acquired using an optical microscope manufactured by Olympus.

For immunofluorescence staining, after antigen retrieval, sections were incubated with a 10% goat serum solution at ambient temperature for a period of 1 hour to block

nonspecific binding. The sections were incubated with primary antibodies, including anti-NRF2 (Servicebio, Wuhan, China), anti-HO-1 (Servicebio, Wuhan, China), and anti-8-OHDG (Proteintech, Wuhan, China), at 4°C overnight. On the following day, after rinsing with phosphate-buffered saline (PBS), the sections were incubated with the appropriate secondary antibodies at room temperature for 2 hours. Staining was observed using an EVOS® FL automatic cell imaging system (Invitrogen). The region of interest for examination was between the roots of the first and second molars.

2.4. Culture and intervention

The RAW264.7 cell line (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum and 1% antibiotics. The cells were grown at 37°C in a humidified incubator containing 5% CO2.

To simulate an inflammatory environment, the groups and treatments were as follows: control group, no treatment; Hcy group, treatment with 100 nM/mL (0.01% v/v) DL-homocysteine (DL-Hcy, Sigma, Germany) for 12 or 24 hours; LPS group, treatment with 1 μg/mL (0.0001% v/v) *Porphyromonas gingivalis*-derived lipopolysaccharide (LPS, Invivo Gen, France) for 12 or 24 hours; and LPS + Hcy group, 6-hour pretreatment with Hcy and then exposure to LPS for either 12 or 24 hours.

To simulate an osteoclast-inducing environment, the groups and treatments were as follows: control group, no treatment; Hcy group, treated with 100 nM/mL (0.01% v/v) DL-homocysteine (DL-Hcy, Sigma, Germany) for 7 days; Rankl group, stimulated with 50 ng/mL (0.000005% v/v) receptor activator for nuclear factor-κB ligand (Rankl, R&D Systems, USA) for 7 days; and Rankl + Hcy group, cotreated with Rankl and Hcy for 7 days. The culture medium was changed at two-day intervals until the presence of osteoclasts was detected.

2.5. Quantitative reverse transcriptase polymerase chain reaction (qRT–PCR)

Total RNA was extracted from gingival tissues and cells using a suitable extraction method and Monzol™ Reagent Pro (Monad, Suzhou, China) and reverse transcribed using MonScript™ RT ALL-in-One Mix with dsDNase (Monad, Suzhou, China). qRT–PCR was performed using a QuantStudio™ 3 Real-Time PCR Instrument (Thermo Fisher Scientific) and MonAmo™ RapidStart Universal SYBR Green qPCR Mix (Monad, Suzhou, China) under the following conditions: 95°C for 1 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. qRT–PCR experiments were performed in triplicate for cell samples and in sextuplicate for animal samples. The relative expression levels of the genes were calculated using the 2-ΔΔCt method, with GAPDH (for mouse genes) used as the internal reference. The primer sequences are listed in Table 1.

Table 1. Prime sequences of mRNA.

Gene	Forward Primer (5′–3′)	Reverse Primer (5′–3′)
IL-1β	TGAATTGGTCATAGCCCGCA	TCTCCTTCCTGTGCAAACTCT
IL-6	TCCTACCCCAATTTCCAATGCT	AACGCACTAGGTTTGCCGAG
TNF-α	GCCGATGGGTTGTACCTTGT	TCTTGACGGCAGAGAGGAGG
Nrf2	ACACGAGATGAGCTTAGGGC	TCGGATCAATGCGAGCTGAG
HO-1	CTGTCCAGTTGGTGTGGATAA	TCAGGCAGAGGGTGATAGAA
NQO1	CAGTCAAATCTGGTGGCATC	GCTGCAGACCTGGTGATATT
GPX	TCATTTGGTCTCCGGTGTGC	TGTCGATGGTACGAAAGCGG
NFATc-1	CCGTCACATTCTGGTCCATAC	TTCATTCTCCAAGTAACCGTGTAG
CTSK	AATTATGGCTGTGGAGGCGG	TGCATTTAGCTGCCTTTGCC
MMP-9	ATGTCACTTTCCCTTCACCTTC	TGCCGTCCTTATCGTAGTCA
TRAP	CACTCCCACCCTGAGATTTGT	CATCGTCTGCACGGTTCTG
TGF-β	CACTCCCGTGGCTTCTAGTG	CTGGCGAGCCTTAGTTTGGA
IL-10	CCTGGGTGAGAAGCTGAAGAC	CTTGTAGACACCTTGGTCTTGG
Arg-1	CAGCAGAGGAGGTGAAGAGTA	TAGTCAGTCCCTGGCTTATGG
GSH	TCATTTGGTCTCCGGTGTGC	TGTCGATGGTACGAAAGCGG
8-OHdG	CTCTTCGGCCCATGTGTACC	CAGGTTAGACCATGGCGTGT
GAPDH	TGAGGTGACCGCATCTTCTTG	TGGTAACCAGGCGTCCGATA

2.6. Western blot (WB) analysis

To obtain total protein, cultured cells were treated with RIPA buffer (Servicebio, Wuhan, China) supplemented with a protease inhibitor cocktail (Servicebio, Wuhan, China). Appropriate SDS–PAGE gels (10%, Yeasen, Shanghai, China) were used according to the molecular weights of the proteins. After electrophoresis, the proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA). The membranes were incubated with quick blocking solution (ShareBio, Shanghai, China) for 15 minutes at ambient temperature to prevent nonspecific binding and then incubated with primary antibodies against β-Tubulin (1:10,000, Proteintech, Wuhan, China), Nrf2 (1:1,000, Immunoway, Beijing, China), HO-1 (1:1,000, Abcam, Shanghai, China), NQO1 (1:1,000, Zen-bio, Chengdu, China), TNF-α (1:1,000, Proteintech, Wuhan, China), NFATc-1 (1:1,000, Proteintech, Wuhan, China), CTSK (1:1,000, Proteintech, Wuhan, China), Lamin B (1:1,000, Zen-bio, Chengdu, China), and Histone 3 (1:1,000, Zen-bio, Chengdu, China) overnight at 4°C. The following day, after the membranes had been rinsed three times with TBST, they were incubated with the appropriate secondary antibodies (mouse/rabbit) (1:20,000, Proteintech, Wuhan, China) for 2 hours at room temperature. An enhanced chemiluminescence (ECL) detection kit (UElandy, Suzhou, China) was used to visualize the protein bands on the membranes, and protein quantification was carried out using ImageJ software (version 1.8.0). Each protein band was analysed at least three times.

2.7. Enzyme-linked immunosorbent assay (ELISA)

To assess the levels of Hcy in animal serum, a Homocysteine (Hcy) Colorimetric Assay Kit (Elabscience, Wuhan, China) was used following the protocol provided by the manufacturer. The results are expressed in μmol/L.

To measure the concentrations of IL-1β, IL-6, TNF-α, and CRP in animal serum, ELISA kits (SiZhengBo, Beijing, China) were used following the protocol provided by the manufacturer. The absorbance was measured at 450 nm using a Spectra MAX Absorbance Reader CMAX Plus plate reader (Molecular Devices), and the concentrations of IL-1β, IL-6, TNF-α, and CRP were determined using a standard curve. The results are expressed in pg/ml or μg/ml.

2.8. Detection of oxidative stress

Cells were seeded into six-well plates at a concentration of 1×10^5 cells/mL for subsequent analysis. The levels of intracellular reactive oxygen species (ROS) were measured using an ROS detection kit (Beyotime, Shanghai, China). Fluorescence images were observed under a fluorescence

microscope. The level of lipid peroxidation, which reflects the degree of oxidative damage within the cells, was measured using a malondialdehyde (MDA) assay kit (Beyotime, Shanghai, China). Total superoxide dismutase (SOD) activity was measured using the WST-8 method with an SOD activity assay kit (Elabscience, Wuhan, China).

2.9. Statistical analysis

All the data are presented as means ± standard errors of the means (SEMs). Data from a normally distributed population were subsequently analyzed using one-way analysis of variance (ANOVA) for intergroup comparisons, followed by Tukey's test. All the statistical analyses were performed using GraphPad Prism 9.5 software (San Diego, USA), and a *P*-value of less than 0.05 was considered statistically significant. All the experiments were conducted at least three times in triplicate for each condition.

3. Results

3.1. HHcy exacerbates inflammatory responses and bone loss in the alveolar region in periodontitis model mice

We first measured the concentration of Hcy in the serum of model mice (Figure 1A). The results revealed that a high-protein diet (25% casein) effectively increased the serum Hcy level in mice to >15 μmol/L (Figure 1C), indicating the successful induction of HHcy in these mice. Compared with those in the control group, the levels of Hcy in the serum of the mice in the HHcy group and the PD group were both greater, with the PD + HHcy group having the highest Hcy level, suggesting a possible synergistic effect between PD and HHcy. Clinically, the distance from the cementoenamel junction to the alveolar bone crest (CEJ-ABC), which is used to quantify the degree of alveolar bone resorption, is an important indicator for measuring the degree of periodontal attachment loss. Compared with the control group, the PD group presented obvious alveolar bone resorption, and the PD + HHcy group presented further exacerbated alveolar bone resorption (Figure 1B, E); H&E staining revealed similar results (Figure 1D). To assess the systemic and periodontal inflammation levels in the mice, we used ELISAs and qRT–PCR to assess the expression levels of relevant inflammatory factors in the serum and gingival tissues, respectively, of the mice. Compared with those in the control group, the levels of IL-1β, IL-6, TNF-α, and CRP in the serum of the HHcy and PD groups were higher (Figure 1F-I), and the mRNA levels of IL-1β, IL-6, and TNF-α in the gingival tissues followed similar trends (Figure 1J-L), indicating that both HHcy and PD can lead to increased systemic and local inflammation levels and that the PD + HHcy group presented the highest relative expression levels of the aforementioned inflammatory indicators, suggesting a possible synergistic effect between HHcy and PD.

3.2. HHcy exacerbates oxidative damage in mouse periodontal tissues

To assess the level of oxidative damage in mouse periodontal tissues and explore the mechanisms by which HHcy exacerbates periodontitis, we conducted immunohistochemistry (IHC) and immunofluorescence (IF) on mouse periodontal tissues. TRAP staining revealed clear osteoclasts in both the PD and PD + HHcy groups, with an increased number of osteoclasts in the PD + HHcy group compared with the PD group (Figure 2A, B). Compared with that in the control group, the fluorescence intensity of 8-OHdG, a marker for evaluating oxidative tissue damage, was significantly greater in the PD and PD + HHcy groups, indicating that the periodontal tissues were in a state of oxidative damage; moreover, the semiquantitative fluorescence results suggested that the immunofluorescence intensity in the PD + HHcy group was further increased, indicating that HHcy exacerbated the oxidative damage in the periodontal tissues of the PD group (Figure 2C, D). Nrf2, an important protein in the endogenous antioxidant system, can promote the release a series of antioxidant proteins to combat oxidative stress. With respect to the levels of the antioxidant proteins Nrf2 and HO-1 in periodontal tissues, the immunohistochemistry results for Nrf2 indicated a significant decrease in Nrf2 levels in the HHcy and PD groups compared with those in the control group, with further suppression in the PD + HHcy group (Figure 2E, F); immunofluorescence for HO-1 (Figure 2G, H) suggested a trend in fluorescence intensity consistent with that of Nrf2. The mRNA levels of Nrf2, HO-1, and NQO1 in mouse gingival tissues showed the same trend (Figure 2I-K). In addition, compared to the PD group, the results of MDA, SOD, GSSG (Figure 2L, M; Figure S 2A), and the ratio of GSSG/GSH (Figure S2 B) in mouse serum also indicate that the global antioxidant proteins in mice in the HHcy + PD group are suppressed and are in a state of oxidative stress. These results suggest that mice with both PD and HHcy presented the greatest level of oxidative damage and that the expression of key antioxidant-related genes was downregulated.

3.3. Hcy exacerbates inflammatory levels and oxidative damage in RAW264.7 cells

Reactive oxygen species (ROS) are the most direct causes of oxidative stress and oxidative damage in the body. We used a fluorescent probe to detect ROS in RAW264.7 cells and found that pretreatment with Hcy, followed by LPS treatment, resulted in increased levels of reactive oxygen species (Figure 3A–C). The protein content of MDA, GSSG and the ratio of GSSG/GSH (Figure 3D; Figure S 2C, D) exhibited the same trend, whereas that of SOD exhibited the opposite trend (Figure 3E). In the Hcy group and the LPS group, the expression of Nrf2 and its downstream proteins HO-1 and NQO1 was downregulated to a certain extent, and the expression of these antioxidant proteins in the LPS group was further downregulated after pretreatment with Hcy (Figure 3F, H–J). Moreover, the nuclear entry of Nrf2 was inhibited, which was consistent with the trend for total protein (Figure 3G, K). The expression of the antioxidant-related genes Nrf2, HO-1, NQO1, and GPX also exhibited the same trend (Figure 4C–F), whereas the expression of the oxidative damage gene 8-OHdG tended to increase (Figure 4G).

These results indicate that Hcy may exacerbate the degree of oxidative damage in RAW264.7 cells by promoting the release of ROS.

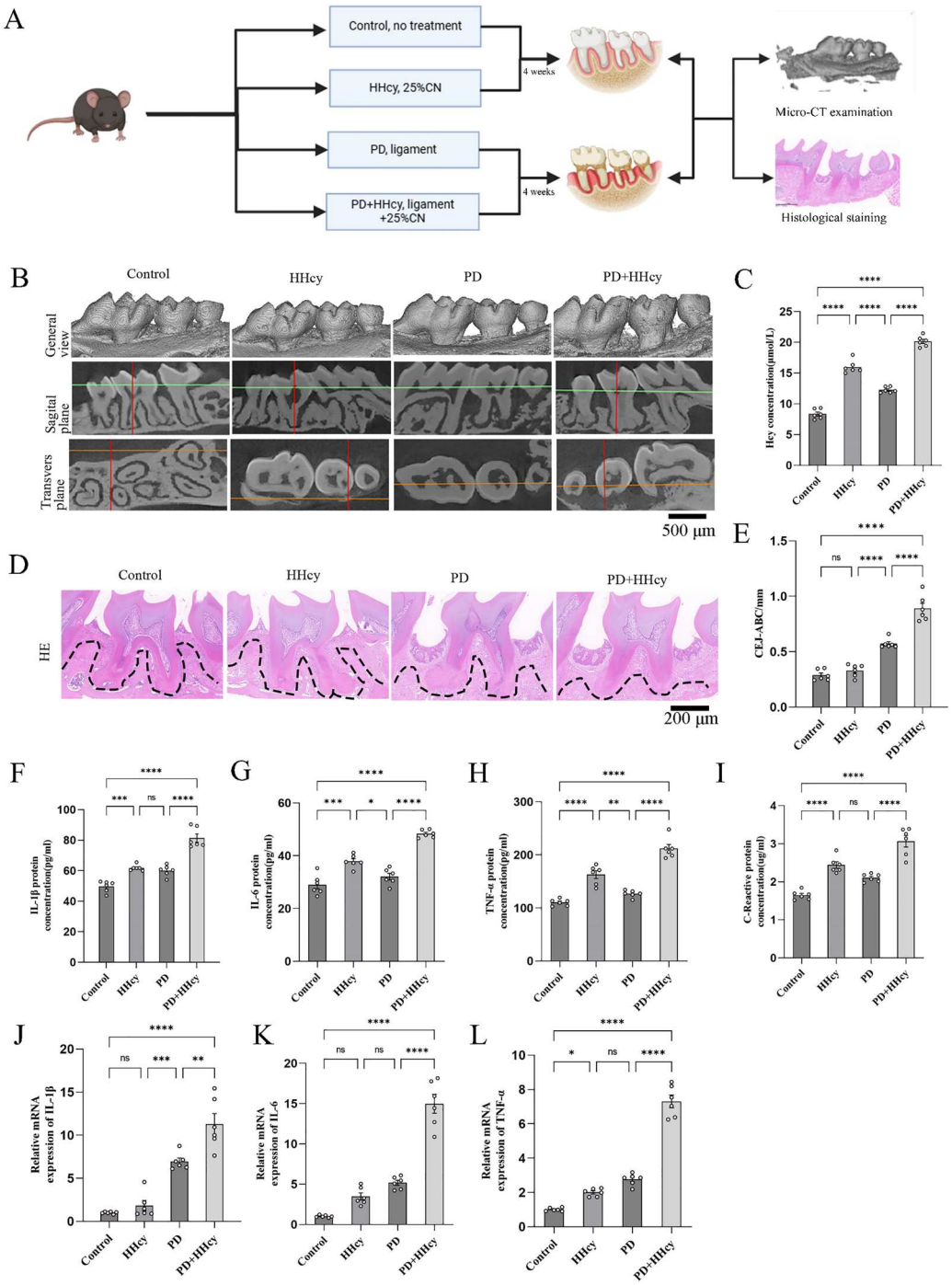

Figure 1. HHcy exacerbates alveolar bone resorption and inflammation levels in mouse periodontitis. (A) Schematic diagram of animal model grouping. (B) Representative Micro-CT images of alveolar bone of periodontitis areas. (Scale bar = 200 μm, n = 3 per group). (C) Hcy concentration in mouse serum. (n = 6 per group). (D) H&E staining of periodontitis areas. (Scale bar = 200 μm, n = 3 per group). (E) CEJ-ABC distance. (F–I) Levels of inflammatory factors IL-1β, IL-6, TNF-α, CRP in mouse serum. (n = 6 per group). (J–L) Relative mRNA expression levels of IL-1β, IL-6, TNF-α in mouse gingival tissue. (n = 6 per group). HHcy, Hyperhomocysteinemia, HHcy; PD, periodontitis. Bar graphs: Values are presented as mean ± SEM. ns, not significant. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. ****$P < 0.0001$.

To assess the inflammatory status of RAW264.7 cells, we assessed the protein level of TNF-α, which tended to be higher in both the Hcy group and the LPS group compared with the control group, and it further increased after pretreatment with Hcy (Figure 4A, B). Compared with the LPS group, the LPS + Hcy group showed significantly upregulated expression levels of pro-inflammatory-related genes IL-1β, IL-6, TNF-α, and MMP-9 (Figure 4H–K), while anti-inflammatory-related genes IL-10, TGF-β, and Arg-1 exhibited a significant downward trend in expression (Figure 4L–N).

The above results illustrate that Hcy may induce excessive ROS production, thereby inhibiting the transfer of Nrf2 to the nucleus and subsequently inhibiting the release of downstream antioxidant proteins, exacerbating oxidative damage and inflammatory responses.

3.4. Hcy enhances osteoclastogenesis by exacerbating oxidative damage through the Nrf2/HO-1 signalling pathway in RAW264.7 cells

To explore the effect of Hcy on the ability of monocytes to differentiate into osteoclasts, recombinant Rankl protein was used to induce osteoclastogenesis (Figure 5A). We observed osteoclasts in the Rankl group, with the number of osteoclasts being further increased in the Rankl + Hcy group (Figure 5B, C). At the protein level, the expression of

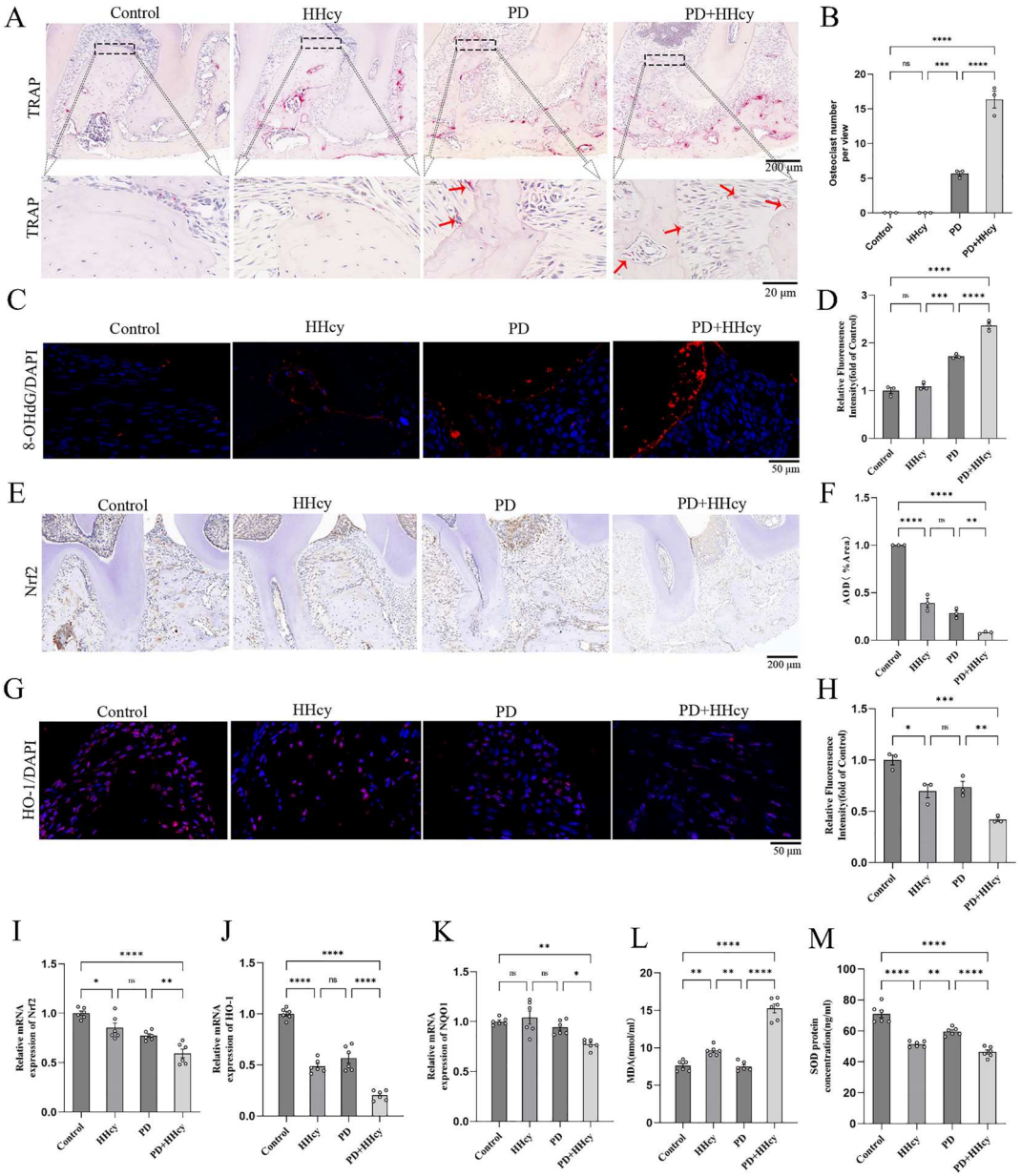

Figure 2. HHcy exacerbates osteoclast damage and oxidative damage levels in mouse periodontal tissues. (A, B) TRAP staining and quantitative analysis in periodontal tissues. Red arrows indicate clear osteoclasts, with TRAP-positive signals and more than three nuclei identified as osteoclasts. (Scale bar = 200, 20 μm, n = 3 per group). (C, D) Representative images of 8-OHDG IF staining in periodontal tissues. (Scale bar = 200, 50 μm, n = 3 per group). (E, F) Representative images of Nrf2 IHC staining in periodontal tissues. (Scale bar = 200, 50 μm, n = 3 per group). (G, H) Representative images of HO-1 IF staining in periodontal tissues and semi-quantitative analysis. (Scale bar = 200, 50 μm, n = 3 per group). (I–K) Relative mRNA expression levels of Nrf2, HO-1, and NQO1 in mouse gingival tissues. (n = 6 per group). (L, M) Levels of oxidative stress-related proteins MDA and SOD in mouse serum. (n = 6 per group). Bar graphs: Values are presented as mean ± SEM. ns, not significant. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. ****$P < 0.0001$.

the osteoclast-related proteins NFATc-1 and CTSK tended to be associated with the osteoclast phenotype (Figure 5D–F). At the mRNA level, NFATc-1, CTSK, TRAP, and MMP-9 presented similar results (Figure 5G–J).

These results indicate that Hcy may inhibit the translocation of Nrf2 to the nucleus, thereby inhibiting the release of antioxidant proteins and enhancing the osteoclast differentiation function of monocytes.

To investigate whether the effect of Hcy on osteoclastogenesis is related to oxidative damage in RAW264.7 cells, the expression of antioxidant-related proteins in monocyte osteoclasts was assessed. In the Hcy group and the Rankl group, the expression of Nrf2 and its downstream proteins HO-1 and NQO1 was downregulated to a certain extent, and Nrf2 expression was further downregulated in the Rankl + Hcy group (Figure 6A, C-E). Moreover, the nuclear translocation of Nrf2 was significantly inhibited, which was consistent with the trend for total protein (Figure 6B, F). Compared to the Rankl group, the mRNA levels of antioxidant-related genes in the Rankl + Hcy group, including Nrf2, HO-1, NQO1, GPX, and GSH, also showed downregulation trend (Figure 6G–K), and the mRNA level of the oxidative damage-related gene 8-OHdG gradually increased (Figure 6L).

4. Discussion

Periodontitis is a chronic inflammatory disease with a complex origin. In 2019, the global incidence of periodontitis was 17%, with more than 1.1 billion individuals having severe periodontitis [22]. Research indicates an association between periodontitis and systemic disorders, including cardiovascular

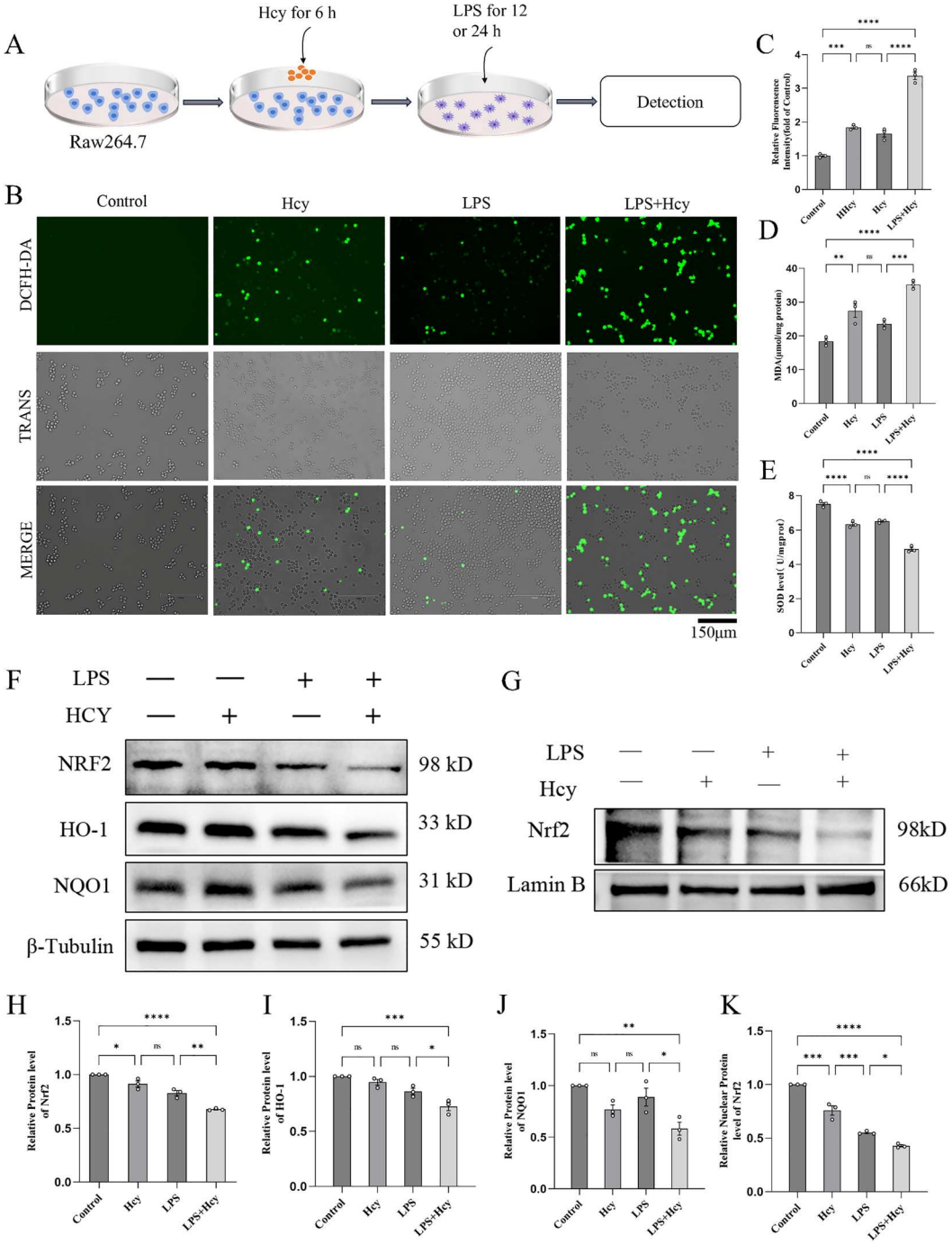

Figure 3. Hcy promotes the release of ROS and oxidative damage in macrophages in vitro, while suppressing the Nrf2/HO-1 signaling pathway. (A) Schematic diagram of LPS + Hcy treatment conditions. (B, C) Fluorescence images and semi-quantitative analysis of intracellular ROS in RAW 264.7 cells stained with DCFH-DA. (Scale bar = 150 μm). (D, E) Protein content of MDA and SOD in RAW 264.7 cells after treatment (n = 3 per group). (F, H–J) Relative total protein expression levels and quantitative analysis of antioxidant proteins Nrf2, HO-1, NQO1 (n = 3 per group). (G, K) Relative nuclear protein expression levels and quantitative analysis of relative protein expression level of Nrf2 (n = 3 per group). Bar graphs: Values are presented as mean ± SEM. ns, not significant. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. ****$P < 0.0001$.

disease, Alzheimer's disease, and type 2 diabetes, with mutual effects [23,24]. Homocysteine is synthesized from the cleavage of the amino acid methionine and is an intermediate metabolic product formed through the conversion of methionine to cysteine. Recent research has revealed a connection between higher Hcy levels and periodontitis development [25,26]; nevertheless, most studies have been restricted to epidemiological surveys.

Among all the experimental animals, the mice in the PD + HHcy group presented the highest serum Hcy levels and the most severe periodontal tissue destruction, suggesting a possible synergistic effect between periodontitis and HHcy [27]. On the one hand, periodontitis may promote further increases in serum Hcy levels by inducing a systemic inflammatory state. On the other hand, HHcy may disrupt the periodontal microenvironment through systemic and local fluid circulation. In vitro experimental results revealed that RAW264.7 cells treated with Hcy presented increased levels of inflammatory factor expression and increased osteoclast differentiation ability in response to LPS and RANKL stimulation. These results corroborate each other, adding credibility to the mutually promoting relationship between HHcy and periodontitis. Our experimental results suggest that HHcy may exacerbate periodontal tissue inflammation by

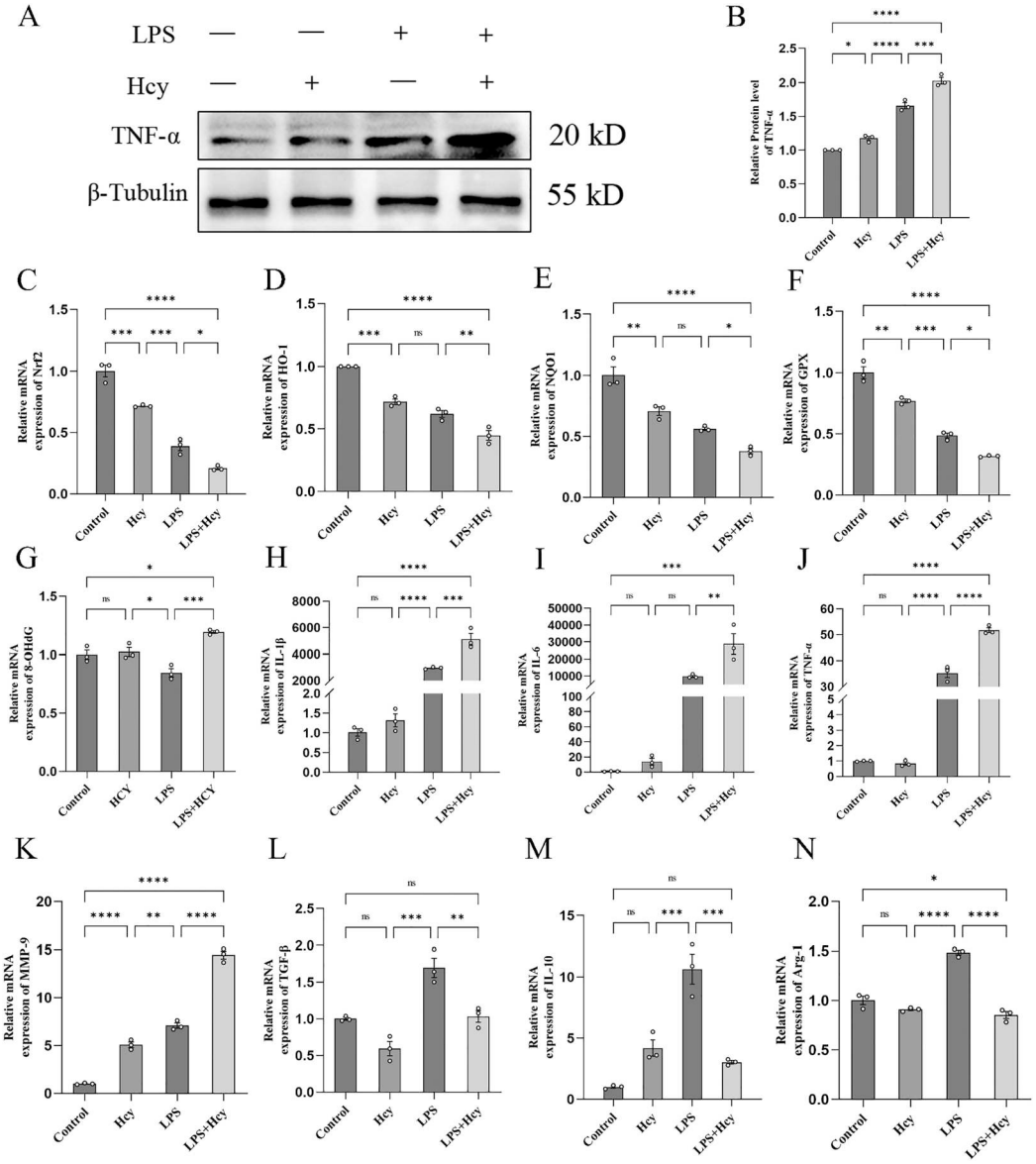

Figure 4. Hcy promotes the expression of pro-inflammatory genes in macrophages under inflammatory conditions in vitro, while suppressing the expression of antioxidant and anti-inflammatory genes. (A, B) Relative total protein expression levels and quantitative analysis of the inflammatory protein TNF-α. (C–G) Relative mRNA expression levels of antioxidant genes Nrf2, HO-1, NQO1, GPX, and the oxidative damage gene 8-OHdG. (H–K) Relative mRNA expression levels of pro-inflammatory related genes IL-1β, IL-6, TNF-α, MMP-9. (L–N) Relative mRNA expression levels of anti-inflammatory related genes IL-10, TGF-β, Arg-1. n = 3 per group. Bar graphs: Values are presented as mean ± SEM. ns, not significant. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. ****$P < 0.0001$.

aggravating local immune-inflammatory responses and promoting osteoclast differentiation, leading to more severe periodontal destruction. This study is also the first to explore the mechanisms by which HHcy promotes periodontal inflammatory responses and alveolar bone recession in both an animal model and at the molecular level, providing a new theoretical basis for further elucidating the relationship between periodontitis and HHcy.

The mechanism by which HHcy causes oxidative damage to the body has been established in systemic diseases, especially cardiovascular diseases [28–30]. When the body experiences oxidative stress, Nrf2, the primary regulatory factor for cellular defence against oxidative stress, activates numerous downstream antioxidant enzymes to preserve the redox balance in the body [31,32]. Our animal experiments revealed that the protein expression of Nrf2 in the periodontal tissues of both the HHcy group and the PD group was inhibited, indicating that mice in the HHcy group and the PD group were in a state of oxidative damage. Immunofluorescence staining of the oxidative damage protein 8-OHdG and the antioxidant protein HO-1 led to the same conclusion, with mice in the PD + HHcy group showing the most significant level of oxidative damage, indicating that HHcy may exacerbate oxidative damage in mouse periodontal tissues by inhibiting the upregulation of Nrf2 expression. In vitro experiments revealed that RAW264.7 cells treated with Hcy under LPS stimulation release increased levels of ROS, and when the body cannot maintain redox balance, ROS act as signalling molecules, inducing the production of inflammatory and osteoclast factors, thus increasing the expression of inflammatory factors and increasing the capacity for osteoclast differentiation.

Our results revealed that serum Hcy levels in animal models are consistent with the levels of C-reactive protein. C-reactive protein, a sensitive indicator of inflammation, infection, and immune diseases in the body, is related to the severity of periodontitis [33,34]. Although more research and confirmation are needed to determine the precise mechanism, we hypothesize that HHcy may worsen periodontitis

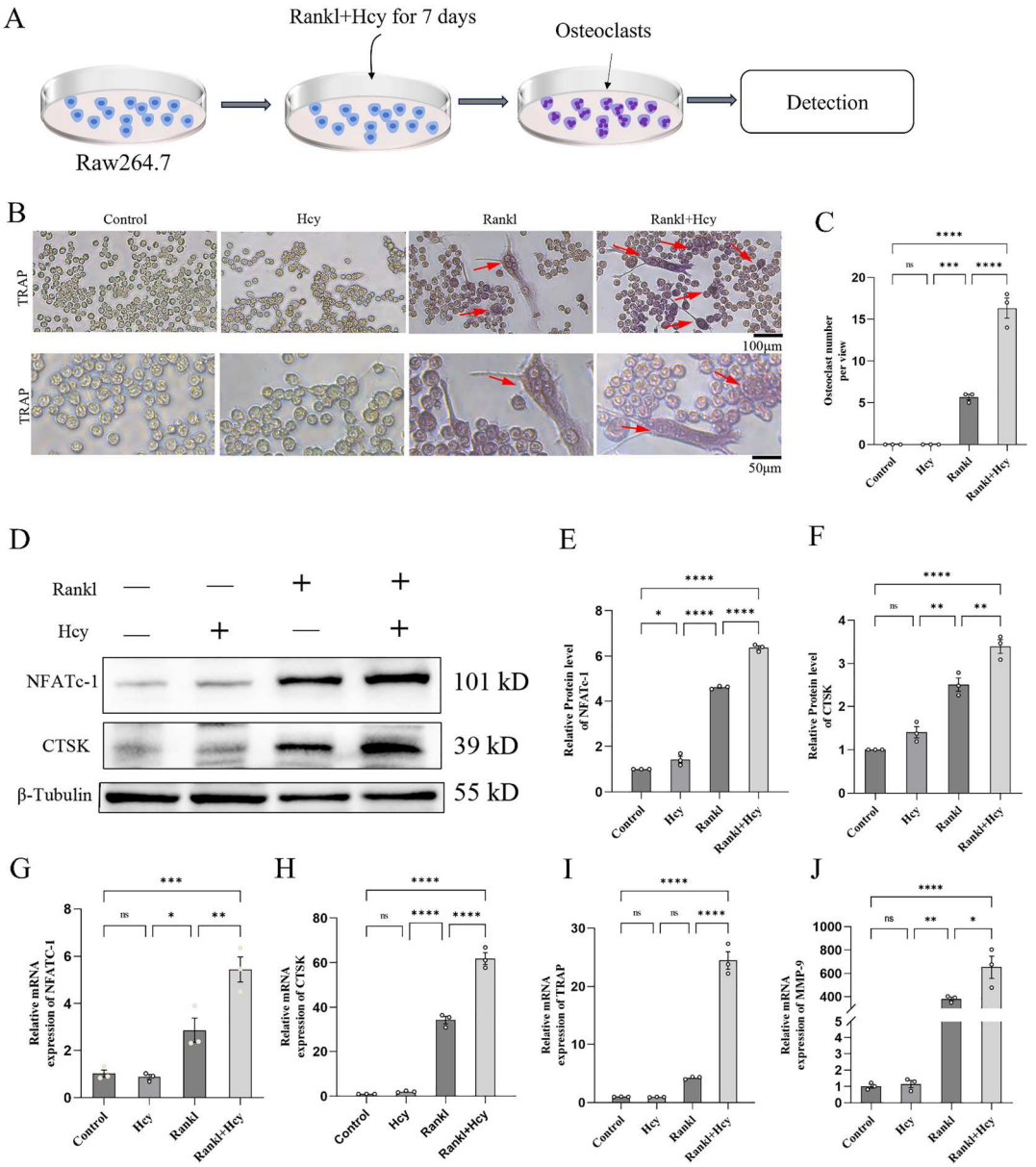

Figure 5. Hcy promotes osteoclast differentiation and the expression of osteoclast-related proteins in monocyte-derived osteoclasts. (A) Schematic diagram of treatment conditions for the Rankl + Hcy group. (B, C) Phenotype and quantitative analysis of the osteoclast differentiation ability promoted by Hcy. Red arrows indicate obvious osteoclasts, with TRAP-positive signals and more than three nuclei identified as osteoclasts. (Scale bar = 100, 50 μm). (D–F) Expression and quantitative analysis of osteoclast-related proteins NFATc-1, CTSK. (G–J) Relative mRNA expression levels of osteoclast-related genes NFATc-1, CTSK, TRAP, MMP-9. n = 3 per group. Bar graphs: Values are presented as mean ± SEM. ns, not significant. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. ****$P < 0.0001$.

through the secretion and release of C-reactive protein. Folic acid intake is the preferred strategy for treating HHcy [35,36]. Studies have shown that after periodontal scaling and root planning treatment, the systemic intake of folic acid plays a positive role in periodontal treatment and can reduce the levels of Hcy and C-reactive protein in the gingival crevicular fluid of patients with periodontitis. This further provides clinical evidence that lowering Hcy levels with folic acid helps with periodontal treatment [8]. As a future research goal and direction, we would like to investigate the changes in oxidative stress-related indicators in periodontitis models with folic acid as an Hcy inhibitor.

Considering that both HHcy and periodontitis are closely related to systemic diseases, including cardiovascular diseases, Alzheimer's disease, type 2 diabetes, and other diseases, we considered whether the level of Hcy may be an intermediary factor in establishing a connection between periodontitis and systemic diseases; this has been reported in a recent study [37,38], but more clinical data and experimental data are needed to clarify the relationships among the three.

However, this study also has certain limitations. The research only explored the effects of Hcy on macrophage cell lines and has not yet assessed its impact on other cells in periodontal tissues, such as periodontal ligament stem cells and gingival fibroblasts. Furthermore, the mechanism by which Hcy affects periodontitis requires further in-depth investigation. It is also worth considering whether Hcy has an impact on endoplasmic reticulum stress and epigenetic modifications, in addition to oxidative stress. We will further explore these limitations in subsequent experiments.

In summary, HHcy was found for the first time to exacerbate oxidative damage and osteoclast differentiation in

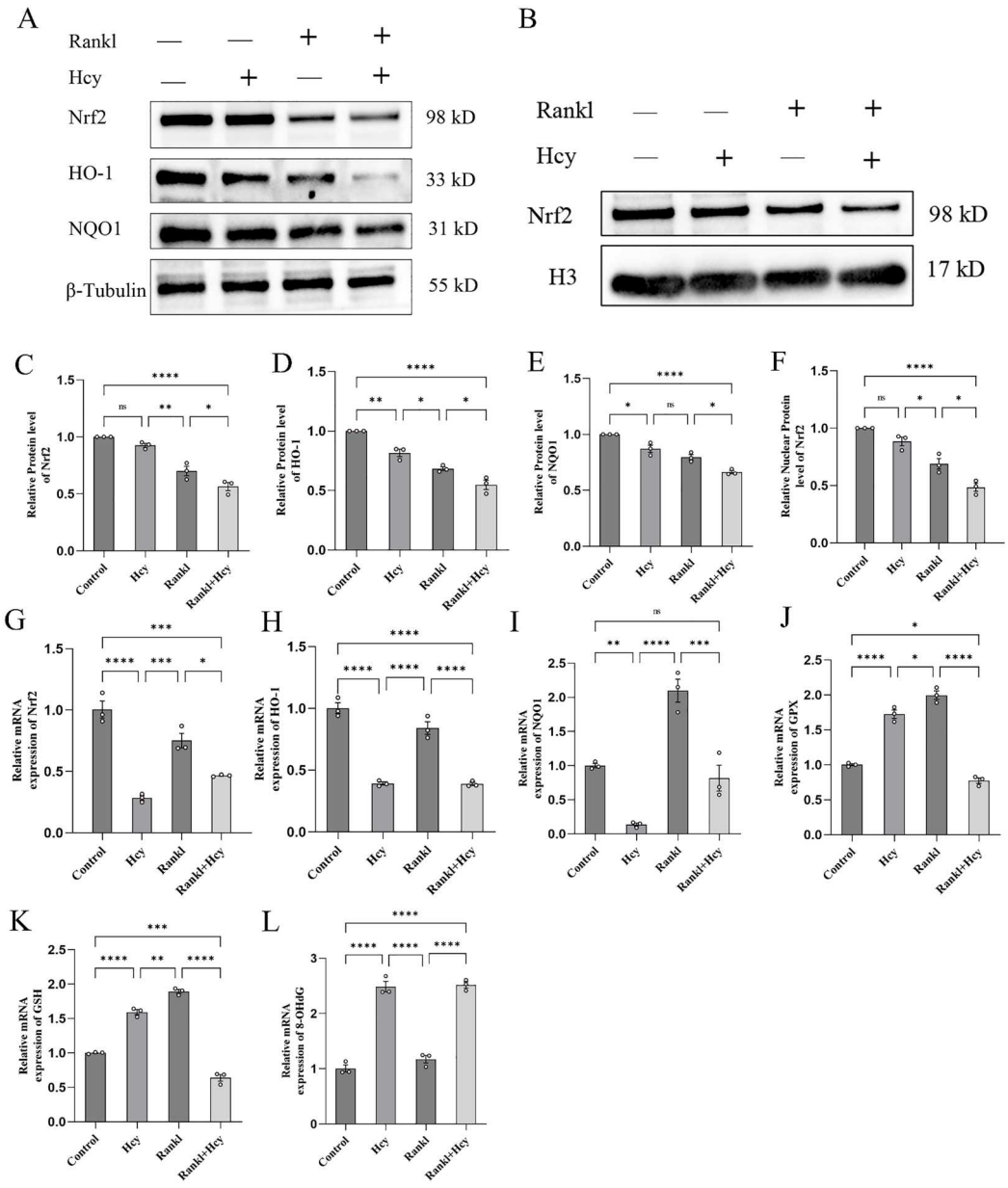

Figure 6. Hcy inhibits the expression of antioxidant proteins and genes in monocyte osteoclasts. (A, C-E) Relative total protein expression levels and quantitative analysis of antioxidant proteins Nrf2, HO-1, NQO1. (B, F) Relative nuclear protein expression levels and quantitative analysis of relative protein expression level of Nrf2. (G-K) Relative mRNA expression levels of antioxidant-related genes Nrf2, HO-1, NQO1, GPX, GSH. (L) Relative mRNA expression level of oxidative damage-related gene 8-OHdG. n = 3 per group. Bar graphs: Values are presented as mean ± SEM. ns, not significant. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. ****$P < 0.0001$.

periodontal tissues by inhibiting the Nrf2/HO-1 antioxidant pathway. It is anticipated that in the future, Hcy will serve as a risk indicator for both the prevention and development of periodontitis because Hcy levels are strongly linked to the degree of periodontal tissue damage.

5. Conclusion

The results of this study confirm that HHcy exacerbates inflammation and attachment loss in mice with periodontitis, reduces antioxidant levels both systemically and locally in mice, and increases the levels of oxidative stress both systemically and locally. This damaging effect is closely related to Hcy promoting the massive release of inflammatory factors by macrophages and accelerating the generation of osteoclasts. The promoting effect of Hcy on inflammation and osteoclastogenesis may act through the inhibition of the Nrf2/HO-1 signalling pathway. A limitation of this study is the lack of effective therapeutic methods to eliminate the effects of Hcy and further verify its impact on Nrf2/HO-1. To further clarify the interaction between HHcy and periodontal disease (PD), more clinical experiments are needed.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Competing interest

The authors report there are no competing interests to declare.

Data availability statement

All data associated with this study are present in the paper. Any information for this study is available by contacting the corresponding authors upon reasonable request.

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