Journal of Dental Sciences 19 (2024) 2106–2113

Available online at www.sciencedirect.com

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journal homepage: www.e-jds.com

Original Article

Prevention of medication-related osteonecrosis of the jaw in mice by adipose-derived stem cells associated with activated autophagic flux

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Received 8 April 2024; Final revision received 5 May 2024
Available online 22 May 2024

KEYWORDS
Autophagy;
Adipose-derived stem cells;
Gingival epithelium;
Medication-related osteonecrosis of the jaw;
Mice

Abstract *Background/purpose:* Medication-related osteonecrosis of the jaw (MRONJ) represents a rare yet serious adverse reaction associated with the prolonged use of anti-bone resorptive or anti-angiogenic agents. This study aimed to investigate the impact and underlying mechanisms of adipose-derived stem cells (ADSCs) in preventing MRONJ in a mouse model.
Materials and methods: Following tooth extraction in MRONJ mice, ADSCs or PBS were administered via the tail vein. The healing progress of gingival epithelium and the extraction socket was assessed using a stereoscopic microscope and histological analysis. Immunofluorescence was employed to examine markers associated with autophagy (LC3 and SQSTM1) and apoptosis (Cleaved-CASP 3). Statistical analysis involved unpaired Student's t-test and ANOVA on ABI Prism 7500, with P-values below 0.05 deemed statistically significant.
Results: ADSCs enhanced gingival epithelium migration and facilitated new bone formation. In the MRONJ group, the expressions of autophagy-related protein LC3 and SQSTM1 in gingival epithelium were concurrently elevated, which indicated autophagic flux was impaired. Conversely, when treated with ADSCs, the expression of LC3 and SQSTM1 were downregulated, similarly to the Control group. Mechanically, zoledronate induced a deficiency of autophagosome–lysosome fusion in epithelial cells, while ADSCs supernatant could promote

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https://doi.org/10.1016/j.jds.2024.05.003
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the autolysosomes formation. Furthermore, ADSCs rescued the number of autophagy-related apoptotic cells in the gingival epithelium of MRONJ.
Conclusion: ADSCs could effectively prevent the occurrence of MRONJ, likely through the activation of autophagic flux and the inhibition of autophagy-related apoptosis in gingival epithelium. These findings enhanced the understanding of MRONJ pathogenesis and propose a potential therapeutic target for this disease.
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Introduction

Medication-related osteonecrosis of the jaw (MRONJ) is a disease with a low incidence, but significantly affects patients' quality of life.[1,2] Surgery has been the primary treatment method, with a recurrence rate of approximately 20%.[3] Adipose-derived stem cells (ADSCs) may offer a promising alternative for intervening in MRONJ.[4–6] In our prior study, we successfully established an MRONJ animal model using New Zealand rabbits and confirmed that ADSCs can prevent MRONJ by promoting primary gingival healing.[7] However, the relevant mechanism by which ADSCs prevent MRONJ remains unclear. Exploring this mechanism would provide a crucial theoretical basis for the future clinical application of ADSCs.

Macroautophagy/autophagy, is a vital biological process for recycling cell components by removing damaged cell organelles and protein aggregates. It assists cells in maintaining a dynamic balance in synthesis, degradation, and subsequent cycles.[8,9] Autophagosome marker LC3 and autophagy-key protein BECN1 persistently express from E16.5 to adulthood in mouse skin epithelium.[10] Furthermore, autophagy deficiency in keratinocytes not only downregulated their proliferation and migration abilities but also inhibited fibroblasts activation, leading to delayed wound healing in mice skin.[11] Collectively, these studies highlight the pivotal role of autophagy in epithelial development, regeneration, and homeostasis.

MRONJ gingival lesions also exhibit delayed epithelial healing. However, whether ADSCs could prevent MRONJ through an autophagy-related mechanism has not been reported. In this study, we explored the effect of ADSCs in preventing MRONJ in a mouse model, possibly through activating autophagy and inhibiting apoptosis. These findings enhance our understanding of the pathogenesis of MRONJ and propose a potential therapeutic target for this disease.

Materials and methods

Animal model and treatment of medication-related osteonecrosis of the jaw

Male C57BL/6N mice (6 weeks old) were procured from Beijing Vital River Laboratory Animal Technology Co., Ltd. These animals were housed in specific pathogen-free conditions, and all procedures were conducted in compliance with the regulations and guidelines of the Ethics Committee of Peking University Health Science Center (approval code: LA2022414). The MRONJ animal model was induced as our previously described.[12] Individual C57BL/6N mice received intraperitoneal (i.p.) injections of 1 μg/mg zoledronic acid (ZA, Sigma—Aldrich, St.Louis, MO, USA) once a day until euthanasia. Two weeks after the i.p. injection, the maxillary first molars were extracted.

For treatment, mice were randomly allocated to three groups ($n = 10$/group). (1) Control group: mice received i.p. injections of saline and intravenous PBS after tooth extraction. (2) MRONJ group: mice received i.p. injections of ZA and intravenous PBS after tooth extraction. (3) ADSC group: mice received i.p. injections of ZA and 1.0×10^6 ADSCs, resuspended in 100 μL PBS (ZSGB-BIO, Beijing, China), intravenously after tooth extraction.

Cell culture and treatment

ADSCs were isolated as previously described[4] and approved by the Ethics Committee of Peking University School and Hospital of Stomatology (approval code: PKUSSIRB-201948106). ADSCs were cultured in alpha-MEM (Gibco BRL, Grand Island, NY, USA) with 10% FBS (Sigma—Aldrich), 1% penicillin/streptomycin (Gibco BRL), 2 mM L-glutamine (Gibco BRL), and 10 mM L-ascorbic acid (Sigma—Aldrich) at 37 °C.

HaCaT cells (provided by the central laboratory of Peking University School and Hospital of Stomatology) were maintained in DMEM (Gibco BRL) with 10% FBS and 1% penicillin/streptomycin at 37 °C.

The RFP-GFP-LC3 virus (GENE, Shanghai, China) was transfected into HaCaT cells with a MOI of 20. After 12 h of transfection, the transfected medium was replaced with complete medium. After 72 h, cells were treated with 2 μg/mL Puromycin for 3 days to purify transfected cells. Subsequently, the transfected cells were treated with ZA and observed under a fluorescence microscope.

HaCaT cells were seeded on coverslips and treated with 25 uM ZA for 24 h. After washing with PBS, coverslips were fixed with 4% paraformaldehyde for 20 min and then blocked with goat serum. The primary antibody was incubated overnight at 4 °C, followed by incubation with the

secondary antibody for 1 h at room temperature, prior to DAPI (ZSGB-BIO) counterstaining.

Adipose-derived stem cells transplantation

ADSCs were cultured up to passages 3–5, digested, resuspended in serum-free medium, centrifuged, repeated twice, and counted after resuspension. The cell density was diluted to 1.0×10^6/100 μL with PBS and placed on ice immediately for later use.

Adipose-derived stem cells conditioned medium (ADSCs -CM) collection

ADSCs were cultured up to passages 3–5. When the cell confluence reached 70%, they underwent triple washing with PBS, followed by replacement of the medium with serum-free medium. After 48 h, the supernatant was collected and centrifuged at 300×*g* for 5 min. The supernatant was then subjected to centrifugation with a 30 kDa ultrafiltration tube for 30 min, filtered through a 0.22 μm bacteria filter, and stored at −80 °C.

Histological evaluations

Maxillae were collected, fixed in 10% neutral buffered-formalin, decalcified in 10% EDTA, and embedded in paraffin. Biopsy sections (4 μm) underwent deparaffinization, rehydration, and staining. Hematoxylin and eosin (H&E), Masson's trichrome staining (Solarbio, Beijing, China), and TUNEL staining (Roche, Basel, Switzerland) were performed following the manufacturers' instructions.

For immunofluorescence staining, sections were subjected to antigen retrieval after deparaffinization and rehydration. Subsequently, the sections were treated with goat serum (ZSGB-BIO). Primary antibodies (1:200 dilution) were incubated at 4 °C overnight, followed by secondary antibodies (1:200 dilution) at room temperature for 1 h. The primary antibodies included LC3 (Abcam, Cambridge, UK), SQSTM1/p62 (Cell Signaling Technology, Danvers, MA, USA), and Cleaved-CASP 3 (Cell Signaling Technology). The secondary antibodies included Alexa Fluor 488 (Abcam) and Rhodamine-conjugated antibody (ZSGB-BIO). DAPI was used for nuclear staining. Images were captured with a microscope (Olympus, Tokyo, Japan) and analyzed with Image-Pro Plus (Media Cybernetics, Rockville, MD, USA).

Western blot

Cells were harvested with RIPA buffer (Solarbio) containing protease inhibitors (Thermo Scientific, Waltham, MA, USA). 30 μg protein samples were loaded and separated by 10% SDS-PAGE. Primary antibodies (1:1000 dilution) were incubated overnight at 4 °C. Primary antibodies included LC3 (Abcam), SQSTM1/p62 (Cell Signaling Technologies), BAX (Cell Signaling Technologies), CASP 3 (Cell Signaling Technologies), Cleaved-CASP 3 (Cell Signaling Technologies), and cleaved PARP (Cell Signaling Technologies). Secondary antibodies (1:10,000 dilution, ZSGB-BIO) were incubated with the membranes at room temperature for 1 h. The membrane was visualized on an electrochemiluminescence detection system.

Flow cytometry

HaCaT cells were cultured in a six-well plate for 12 h and then treated with 25 μM ZA for 48 h. The cells were digested with EDTA-free enzyme, washed twice with PBS, collected, and resuspended at a concentration of 1×10^7 cells/mL in Annexin V binding buffer. 100 μL of cell suspension was added to a 5 mL test tube. Annexin V-APC (5 μL) and 7-AAD (5 μL) (Biolegend, San Diego, CA, USA) were added. The mixture was incubated in the dark at room temperature for 20 min. Then, 400 μL of Annexin V binding buffer was added to each test tube and mixed well. Finally, the results were analyzed using flow cytometry.

Statistical analysis

Statistical analysis involved one-way analysis of variance followed by Bonferroni's multiple comparison post hoc test and unpaired Student's t-test. A P value of less than 0.05 was considered statistically significant.

Results

Adipose-derived stem cells prevent the occurrence of medication-related osteonecrosis of the jaw

The MRONJ mouse model was induced as previously described, with ADSCs injected into the mouse's tail vein immediately after tooth extraction (Fig. 1A). One week later, under a stereoscopic microscope, the ADSCs-treated group exhibited accelerated healing of the gingival epithelium compared to the MRONJ group. The bone exposure rate was notably lower in the ADSCs-treated group (20%) than in the MRONJ group (70%) (Fig. 1B and C). Histologically, H&E staining demonstrated that ADSCs significantly promoted the migration of gingival epithelium, covering the extraction socket, comparable to the Control group (Fig. 1D). Masson staining further confirmed ADSCs treatment was effective in inducing new bone formation compared to the MRONJ group (Fig. 1E). The data collectively indicates that ADSCs effectively prevent the occurrence of MRONJ.

Adipose-derived stem cells activate autophagic flux in gingival epithelium from medication-related osteonecrosis of the jaw

Previous studies highlight the pivotal role of autophagy in epithelial wound healing. Therefore, we investigated whether ADSCs could prevent MRONJ through an autophagy-related process. Immunofluorescence staining of the autophagosome marker LC3 increased in MRONJ gingival epithelium compared with the Control group (Fig. 2A and B). However, increased LC3 levels signify either

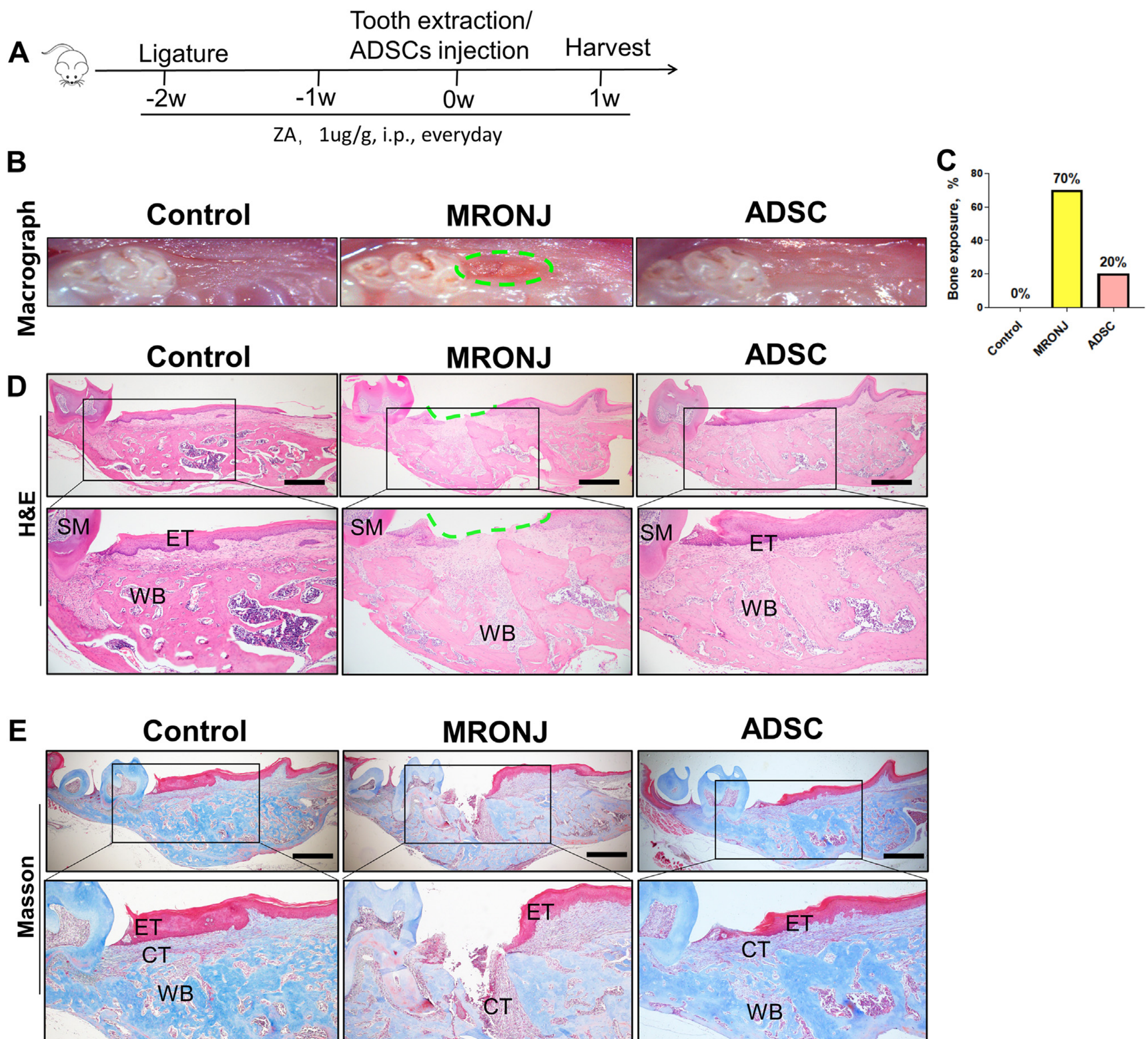

Figure 1 **ADSCs prevent the occurrence of MRONJ.**
(**A**) Experimental design of MRONJ animal model induction. i.p.: intraperitoneal injection. **(B)** Representative images of gingival wounds closure at the extraction sites after one week of tooth extraction. Green circles represent the extraction sites. (C) Quantification of the rate of bone exposure. (**D**) Representative images and magnified boxed regions of H&E staining of wound healing in alveolar sockets. (**E**) Representative images and magnified boxed regions of Masson staining of bone in alveolar sockets. SM: second molar; ET: epithelial tissue; WB: woven bone; CT: connective tissue. Bar: 500 μm.

autophagy induction or deficiency in autophagosome maturation.

We further examined the autophagosome cargo protein SQSTM1/p62, whose accumulation indicating defective autophagy. In comparison with the Control group, both LC3 and SQSTM1 expressions were synchronously upregulated in the MRONJ group (Fig. 2A and B). When treated with ADSCs, the levels of LC3 and SQSTM1 were comparable to the Control group (Fig. 2A and C). These results suggest that autophagic flux was inhibited in MRONJ gingival epithelium, and ADSCs rescued abnormal autophagic flux in MRONJ gingival epithelium.

Adipose-derived stem cells activate autophagic flux via promoting fusion of autophagosomes and lysosomes

To further explore the mechanism of ADSCs activating autophagic flux, we conducted an in vitro experiment using Human keratinocytes cell lines (HaCaT cells). HaCaT cells were transfected with RFP (red fluorescent protein)-GFP (green fluorescent protein)-LC3 lentivirus. Autophagosomes were labeled with both RFP and GFP fluorescence (yellow fluorescence), while autolysosomes were labeled with only RFP fluorescence. There was a significant increase in yellow

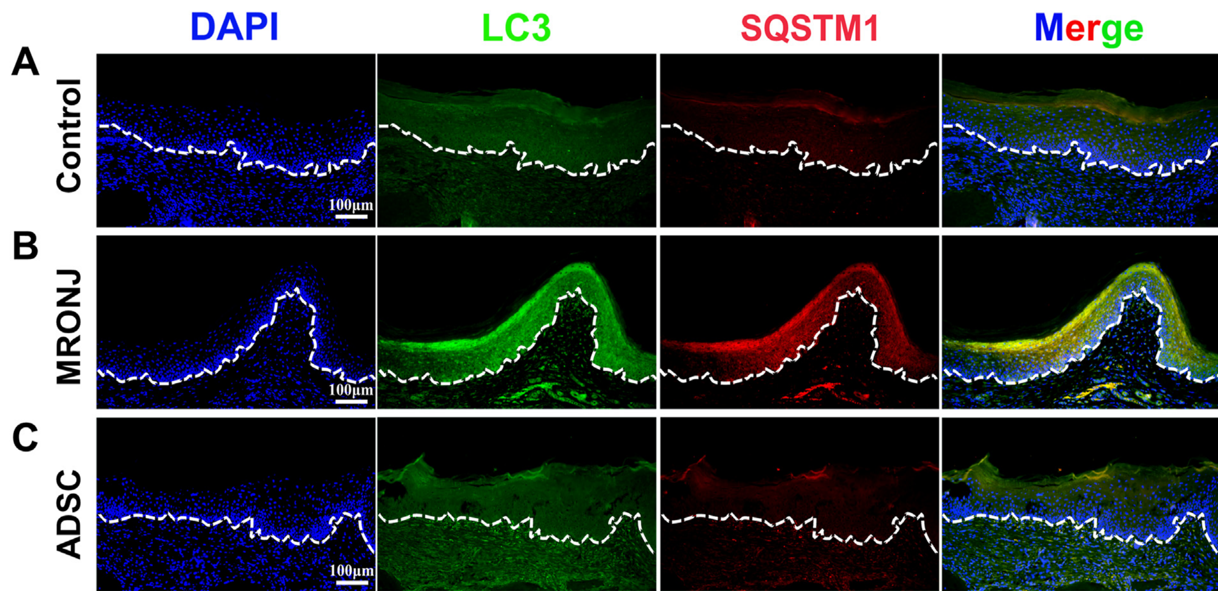

Figure 2 ADSCs activate autophagic flux in MRONJ gingival epithelium.
(**A-C**) Representative immunofluorescence images of gingiva co-stained with antibodies against LC3 and SQSTM1 in the Control, MRONJ and ADSC group. Bar: 100 μm.

puncta in the ZA-treated group compared to the control (Fig. 3A and B), suggesting that ZA led to an autophagosome—lysosome fusion defect.

However, the proportion of yellow fluorescence in HaCaT cells significantly decreased after ADSCs-conditioned medium (CM) treatment (Fig. 3A and B), indicating that ADSCs could promote the fusion of autophagosomes and lysosomes. Western blot showed that the levels of LC3 and SQSTM1 in HaCaT cells treated with ADSCs-CM were rescued compared to those treated with ZA alone (Fig. 3C). In summary, these data suggest that ADSCs can rescue ZA-induced autophagy deficiency by promoting the fusion of autophagosomes and lysosomes.

Adipose-derived stem cells downregulate autophagy-related apoptosis in gingival epithelium from medication-related osteonecrosis of the jaw

Autophagy plays a pivotal role in cell death decisions, offering cellular protection by preventing apoptosis.[13] To assess the role of autophagy in cell fate, we investigated

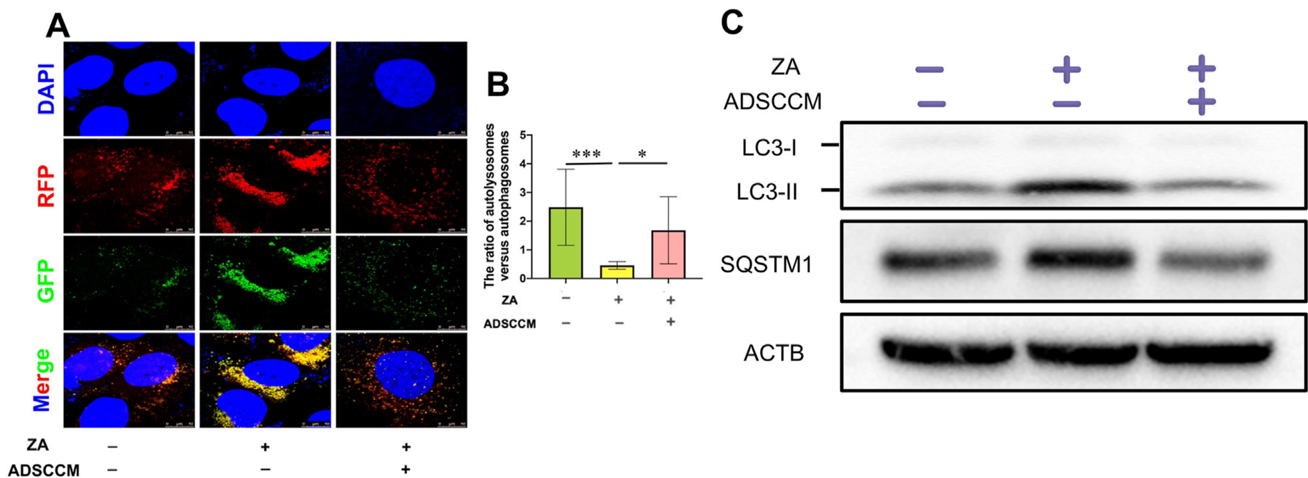

Figure 3 ADSCs activate autophagic flux via promoting fusion of autophagosome and lysosome.
(**A**) Representative images of control, ZA-treated, and ADSCs-conditioned medium (ADSCCM)-treated HaCaT cells expressing RFP-GFP-LC3. Yellow indicates autophagosomes; RFP indicates autolysosomes in cells. (**B**) Quantification of the ratio of autolysosomes versus autophagosomes. (**C**) Western blot of the expression of LC3 and SQSTM1. RFP: red fluorescent protein, GFP: green fluorescent protein. All data are displayed as mean ± SEM. (n = 3 per group). $^{*}P < 0.05$; $^{***}P < 0.005$.

whether increased apoptosis occurred in MRONJ gingival epithelium. Immunofluorescence staining revealed a noticeable increase in Cleaved-CASP 3 in MRONJ gingival epithelium, colocalizing with LC3 (Fig. 4A).

To further assess the impact of ADSCs on gingival epithelial apoptosis, we performed a TUNEL assay on gingival epithelium one week after tooth extraction. The results demonstrated that ADSCs significantly reduced the number of apoptotic cells in the gingival epithelium of MRONJ, with no statistical difference compared to the Control group (Control group 2.261 $\pm$ 0.795%, ZA treatment group 19.305 $\pm$ 4.413%, ADSCs supernatant group 5.581 $\pm$ 1.361%) (Fig. 4B and C).

Adipose-derived stem cells conditioned medium alleviate zoledronic acid-induced HaCaT cells apoptosis

To further confirm whether ADSCs could reverse ZA-induced apoptosis, we utilized ADSCs-CM to treat HaCaT cells stimulated by ZA in vitro. The TUNEL assay demonstrated a greater number of apoptotic cells in the ZA-treated group (26.04 $\pm$ 9.15%) compared to the Control group (1.00 $\pm$ 0.59%), while ADSCs-CM significantly alleviated the number of apoptotic cells (5.42 $\pm$ 5.36%) (Fig. 5A and B). Western blot analysis of apoptosis-related proteins indicated that the upregulation of Cleaved-CASP 3 and cleaved-PARP induced by ZA could be rescued by ADSCs (Fig. 5C). Consistently, flow cytometry showed a significant decrease in apoptotic cells in the ADSCs supernatant-treated group compared to the ZA-treated group (Fig. 5D). In conclusion, ADSCs can effectively alleviate ZA-induced HaCaT cells apoptosis.

Discussion

In this study, we identified impaired autophagic flux associated with the upregulation of apoptosis in gingival epithelial cells as a potential pathogenic mechanism during the development of MRONJ. Additionally, ADSCs demonstrated effectiveness in preventing the occurrence of MRONJ. Taken together, our findings suggest that ADSCs may be a promising candidate for preventing the onset of MRONJ.

Compared with stem cells from other sources, ADSCs are easy to obtain and cause less damage to the donor site,

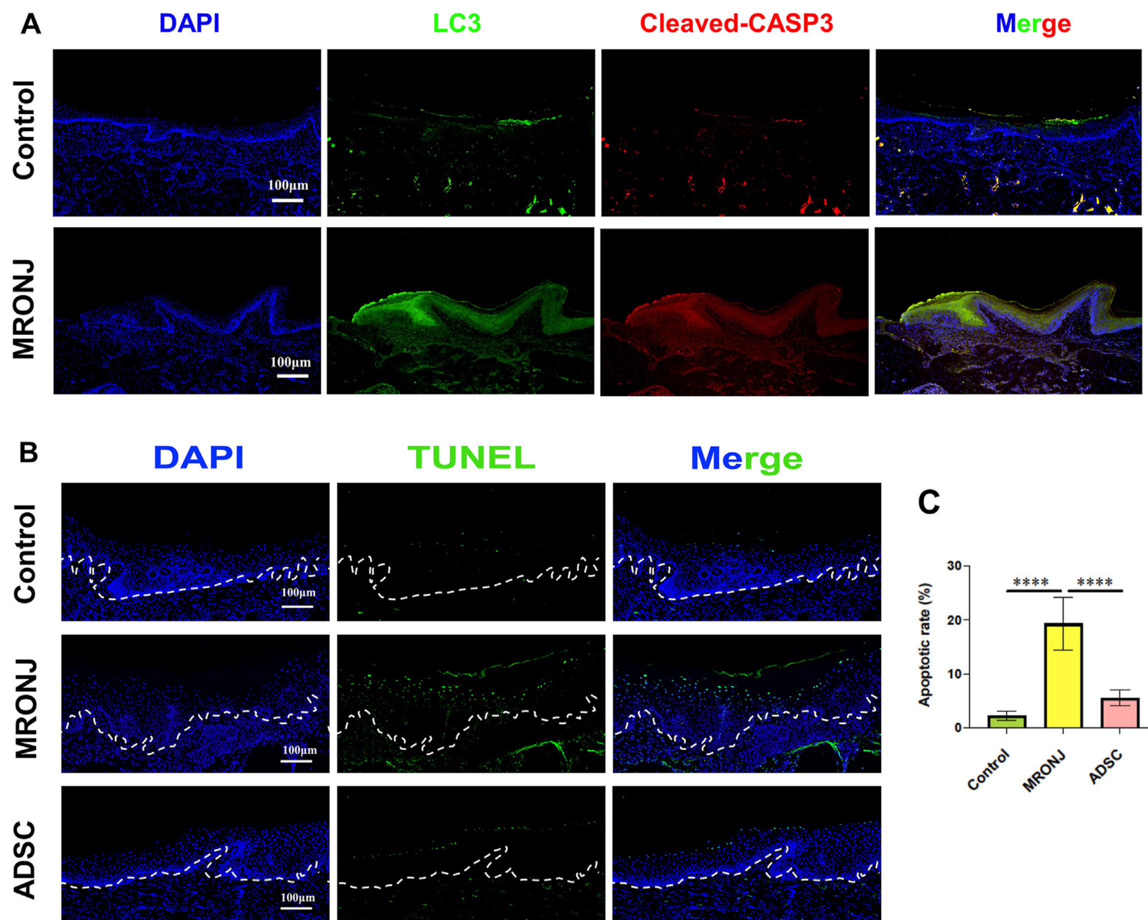

Figure 4 **ADSCs downregulate autophagy-related apoptosis in MRONJ gingiva epithelium.** **(A)** Representative immunofluorescence images of gingiva co-stained with antibodies against LC3 and Cleaved-CASP 3. **(B)** Apoptosis of gingival tissue by TUNEL staining in the Control, MRONJ and ADSC group. **(C)** Quantification of the ratio of apoptotic cells in each group. Bar: 100 μm ****$P < 0.0001$.

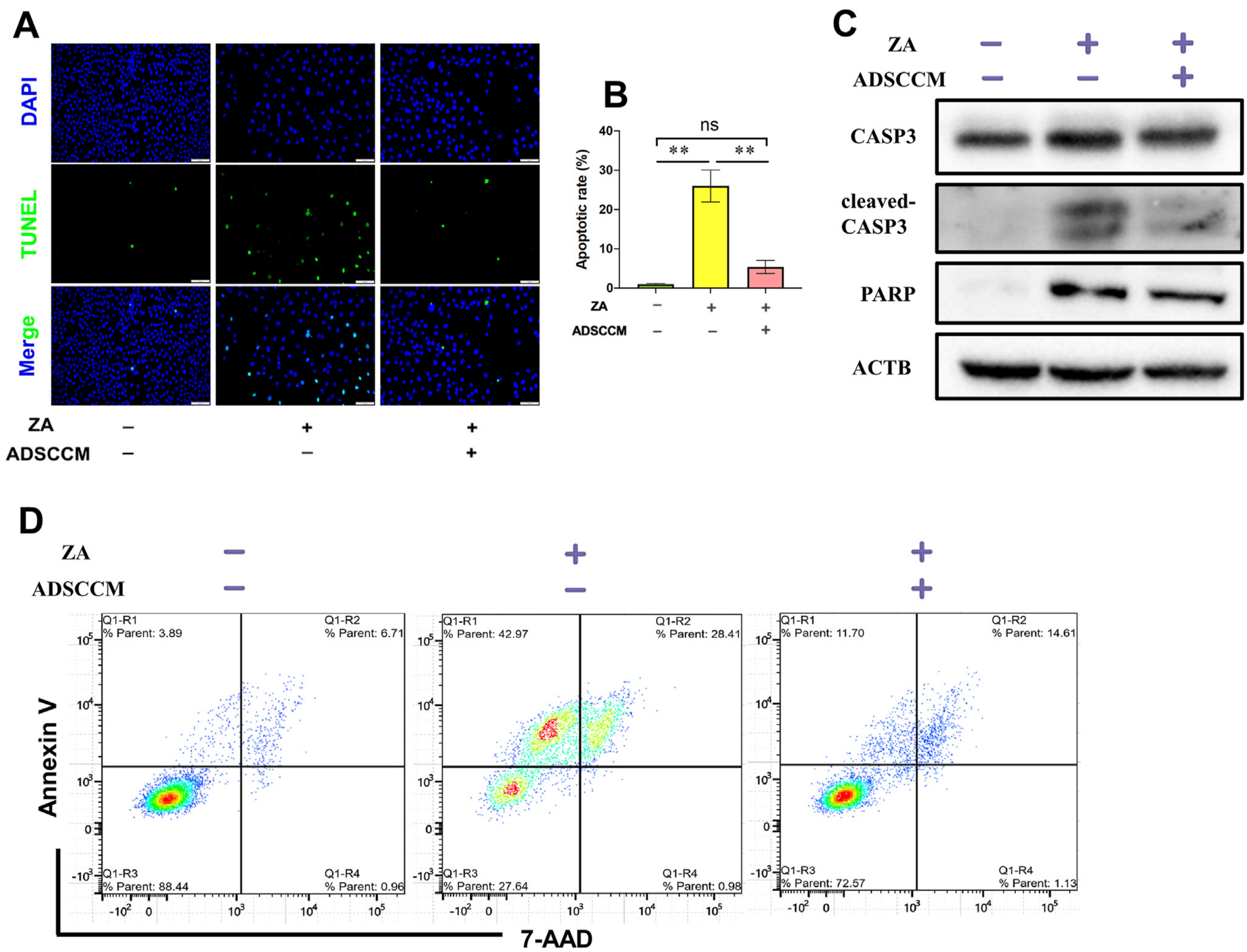

Figure 5 ADSCs-CM alleviate ZA-induced HaCaT cells apoptosis.
(A) Apoptosis of HaCaT cells was assayed by TUNEL staining. (**B**) Quantification of the ratio of apoptotic cells. (**C**) Western blot of the expression of CASP 3, cleaved-CASP 3 and cleaved-PARP. (**D**) Representative plots of Annexin V^{+} and 7-AAD^{+} cells as assessed by flow cytometry. bar: 200um. ns, no significant difference; $**P < 0.01$.

showing broad promising in stem cell therapy.[14,15] In the context of diabetic ulcers, TGF derived from ADSCs effectively promotes re-epithelialization and ulcer healing.[16] ADSCs offer a potential therapeutic intervention in neurodegenerative diseases by shifting microglial phenotype from a classically activated state to an inflammation-resolving one.[17] In our study, the results demonstrated that the administration of ADSCs via the tail vein could prevent the occurrence of MRONJ. Moreover, we found that ADSCs promote soft tissue healing by activating autophagic flux and inhibiting epithelial cell apoptosis.

Autophagy, induced under cellular stress, degrades intracellular accumulated proteins, aged and damaged organelles, and invading pathogenic microorganisms.[18,19] Inhibited or excessive autophagy can induce diseases by interacting with apoptosis, inflammation, and immunity.[20–22] Our study revealed an increased expression of the autophagosome marker LC3 in MRONJ gingival epithelium. Autophagy is a dynamic process with three stages, including autophagosome formation, fusion of autophagosomes and lysosomes, and degradation.[23] The accumulation of SQSTM1, a selective autophagy receptor, colocalizing with LC3 indicates impaired autophagic flux in MRONJ gingival epithelium.

Abnormal autophagic flux may lead to apoptosis. Apoptosis was significantly elevated in MRONJ gingival epithelium in this study. Therefore, the delayed gingival healing in MRONJ may result from the loss of the protective function of autophagy and increased apoptosis during wound healing. The administration of ADSCs via the tail vein significantly down-regulated the expression of key autophagy proteins LC3 and SQSTM1, as well as apoptotic markers in epithelial cells.

Previous studies have demonstrated that mesenchymal stem cells can significantly promote the fusion of autophagosomes and lysosomes, leading to the clearance of β-amyloid protein in Alzheimer's disease animal models.[24,25] Confirming that the deficiency of autophagic flux in epithelial cells is one of the pathogenic factors in gingival epithelial unhealing in MRONJ,[12] we further explored the mechanism of MRONJ prevention by ADSCs. Notably, ADSCs promoted autophagic flux by downregulating the expression

of autophagy key proteins LC3 and SQSTM1 in epithelial cells. These results suggest that ADSCs significantly enhance the fusion of autophagosomes and lysosomes, thus inhibiting apoptosis occurrence. Alternatively, whether ADSCs directly inhibit epithelial cell apoptosis simultaneously, required further investigation.

In conclusion, the study demonstrates that ADSCs can promote the healing of the gingival epithelium in MRONJ mice by enhancing the autophagic flux of epithelial cells and inhibiting apoptosis. These findings may offer a promising treatment avenue for the prevention of MRONJ in the future.

Declaration of competing interest

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

Acknowledgments

We wish to thank the Central laboratory of Peking University School and Hospital of Stomatology for providing the research facilities. The work was supported by Beijing Natural Science Foundation [7222223]; National Natural Science Foundation of China [82101041].

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