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Yunnan Baiyao Inhibits Periodontitis by Suppressing the Autophagic Flux

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ABSTRACT

Background: Yunnan Baiyao (YNBY), a traditional Chinese medicine, is renowned for its anti-inflammatory properties. Recent studies have suggested that YNBY plays a significant role in inhibiting osteoclast differentiation and autophagy, which are essential processes in inflammation and bone resorption associated with periodontitis. However, the precise relationship between autophagy and the mechanism by which YNBY inhibits osteoclastogenesis remains unexplored. The primary objective of this study was to investigate the inhibitory effects of YNBY on the process of osteoclastogenesis and its potential in preventing inflammatory bone loss.

Methods: The animals were subjected to sacrifice at intervals of 2, 4, and 6 weeks postintervention whilst under deep anaesthesia, and specimens were subsequently collected. The specimens were subjected to hematoxylin and eosin (HE) staining, in addition to tartrate-resistant acid phosphatase (TRAP) staining and subsequently imaged employing a digital scanner. The confirmation of osteoclast (OC) differentiation and autophagic flux was achieved through various techniques, including western blotting, transmission electron microscopy (TEM), TRAP staining, pit formation assay, and immunofluorescence.

Results: The microcomputed tomography images provided evidence of the effective inhibition of alveolar bone absorption at 2, 4, and 6 weeks following YNBY treatment. Additionally, the histomorphometric evaluations of tissue segments stained with HE and TRAP, which involved measuring the distance between the alveolar bone crest (ABC) and cemento-enamel junction (CEJ) and quantifying TRAP-positive OCs, yielded comparable results to those obtained through computed tomography analysis. YNBY treatment resulted in a decrease in the CEJ–ABC distance and inhibition of OC differentiation. Furthermore, *in vitro* studies showed that the autophagy modulators rapamycin (RAP) and 3-methyladenine (3-MA) significantly affected OC differentiation and function. YNBY attenuated the impact of RAP on the differentiation of OCs, autophagy-related factor activation, and bone resorption.

Conclusions: We hypothesise that YNBY suppresses the differentiation of OC and bone resorption by blocking autophagy. This study reveals that targeting autophagy might be a new alternative treatment methodology for periodontitis treatment.

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Introduction

Periodontitis is a chronic inflammatory disorder that is linked to the destruction of alveolar bone and periodontal ligament inflammation.¹ Periodontitis is triggered by plaques of

bacteria that cause inflammatory immune reactions, leading to a destruction in the balance between bone formation and resorption.² Numerous investigations have examined the complicated regulatory mechanisms that underlie the loss of alveolar bone. Nevertheless, the pathways remain unidentified regarding periodontitis pathogenesis.

Osteoclasts (OCs), which originate from macrophage-monocyte cells and have multiple nuclei, have a crucial function in the breakdown of bone tissue.³ Excessive development

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and functioning of OCs can result in various conditions connected to bone metabolism, such as periodontitis, osteoarthritis, and osteoporosis.⁴ Focusing on the development and function of OCs is a prominent area of investigation in the study of therapeutic interventions for disorders associated with OCs.

Yunnan Baiyao (YNBY) is a widely recognised constituent of conventional Chinese medicine. At present, YNBY has been observed to cause various effects such as wound-healing, procoagulant, analgesic, anti-inflammatory, antitumour, antibacterial, and other effects.^{5,6} Current investigations have indicated that YNBY exhibits antiarthritic properties through the reduction of the T helper 17 (Th17)/regulatory T cells (Tregs) ratio, regulation of cytokine balance, and repression of OC activity.⁷

Autophagy is an essential intracellular metabolism that regulates various cellular stress responses. The fundamental molecular machinery of autophagy comprises autophagy-associated proteins.^{8,9} Autophagy has been revealed to have a function in OC differentiation and bone resorption according to current studies.^{10,11} However, whether autophagy is related to the inhibitory impact of YNBY on OCs requires further exploration. Therefore, in this investigation, we conducted *in vitro* and *in vivo* trials to investigate the YNBY impact on osteoclastogenesis and a rat model of periodontitis, and we determined which effects are associated with autophagy.

Materials and methods

Reagents and antibodies

M-CSF (#CB34) and RANKL (#CR06) were both acquired from Novoprotein (Shanghai, China). FBS (#E01021) was purchased from Evacell (Hong Kong, China). Alpha-modified MEM (α -MEM) and penicillin-streptomycin were obtained from HyClone (USA). Rabbit polyclonal antibodies against LC3 (#14600-1-AP), ATG5 (#10181-2-AP), Beclin1 (#11306-1-AP), P62 (#18420-1-AP), CTSK (#11239-1-AP), and NFATc-1 (#66963-1-Ig) were acquired from Proteintech Group (Wuhan, China).

Animal model

Sprague Dawley male rats were obtained from the Animal Center, Kunming Medical University (Kunming, China). The investigation was authorised by the Ethics Committee of Kunming Medical University. In order to induce experimental periodontitis, the researchers applied ligatures around the maxillary second molars utilising a 3-0 silk suture with a surgical knot and sustained the ligatures for 4 weeks. Each rat in the periodontitis + YNBY group was administered YNBY (50 μ g/mL). The control group was administered an identical normal saline volume. Animals were euthanised at the end of the experiments with intravenous sodium pentobarbital (100 mg/kg).

Microcomputed tomography (μ -CT)

The animals were subjected to sacrifice at intervals of 2, 4, and 6 weeks postintervention whilst under deep anaesthesia,

and specimens were subsequently collected. Following fixation with 4% paraformaldehyde overnight, μ -CT (NEMO NMC-100, PINGSENG Healthcare Inc.) was employed to scan rat maxillary samples, and the resulting images were subjected to 3-dimensional reconstruction. The linear distance of cemento-enamel junction (CEJ)–alveolar bone crest (ABC) was analysed.

Histomorphometric analysis

The specimens were subjected to hematoxylin and eosin (HE) and tartrate-resistant acid phosphatase (TRAP) staining and subsequently imaged employing a digital scanner (Case Viewer, 3D Histech). The quantification of TRAP-positive cells was performed employing the ImageJ program (NIH).

Cell culture

Female C57BL/6 mouse femurs and tibiae were utilised to isolate bone marrow and collect bone marrow–derived macrophages (BMMs). The cells were induced to undergo osteoclastogenesis by incubation in media treated with 50 ng/mL RANKL and 25 ng/mL M-CSF.

Cell counting kit (CCK)–8 assay

Cell viability assessment was conducted through the utilisation of a CCK-8 cell viability assay (Cell Counting Kit–8, Dojindo, Japan). BMMs were cultivated in 96-well plates with a seeding density of 2×10^3 cells/well. The cells were subjected to supplementation with YNBY (at concentrations of 10, 20, 30, or 40 μ g) or dimethylsulfoxide (DMSO). Following a period of 5 days, the culture medium was substituted with a solution consisting of a 10% CCK-8 mixture. Following 2 hours of incubation, the plates' absorbance was detected at 450 nm by a spectrophotometre. Then, the outcomes were measured.

In vitro osteoclastogenesis assay

The cells were supplemented with 25 ng/mL M-CSF, 50 ng/mL RANKL, and varying concentrations of YNBY (5, 10, or 20 μ g/mL) or DMSO. Following a period of 5 days, the OCs present in the 96-well plates were subjected to fixation through incubation with 4% paraformaldehyde for a duration of 15 to 20 minutes. Subsequently, they were subjected to staining with a TRAP solution for a period of 30 minutes at 37 °C. OCs that exhibited a minimum of 3 nuclei were identified as TRAP-positive, and the quantification of TRAP-positive cells was performed employing the ImageJ program (NIH).

F-actin ring and DAPI staining

The BMMs were cultured in 96-well plates with a seeding density of 1×10^4 cells/well. Subsequently, F-actin ring and DAPI staining procedures were conducted on the 5th day posttreatment. Subsequently, the cells were subjected to treatment with phalloidin for half an hour at room temperature. Subsequently, DAPI staining was conducted for 1 minute under dark conditions at room temperature. Fluorescence microscopy was employed to obtain the images.

Transient transfection with recombinant adenovirus

A particular culture dish suitable for laser confocal microscopy was utilised for seeding the cells. Upon achieving 50% to 80% confluence, the adenovirus was administered to the cells. The adenoviral infection was conducted based on the directions provided by the manufacturer, with a multiplicity of infection value of 100. Following an 8-hour period of infection, the medium was exchanged with a new medium. The cells were subsequently subjected to group-specific treatment for a duration of 6 hours postinfection. Following the administration of treatment, subsequent evaluation and visualisation were performed employing confocal laser scanning microscopy.

LysoTracker Red staining

A red fluorescent probe known as LysoTracker Red, which is utilised for labelling lysosomes, was used to label the lysosomes in living cells. The cell culture methods described above were followed. The medium was replaced with the prepared lysosome staining solution when the cells reached 50% to 70% confluence, and the cells were incubated in an incubator for 6 hours. Next, a microscope was utilised to examine the cells, and the intensity of fluorescence was calculated employing the ImageJ program.

Transmission electron microscopy (TEM)

The utilisation of TEM was employed as the preferred method for the examination of autophagosome generation. In summary, the cells underwent a series of procedures, including washing with PBS, fixing with electron microscope fixing solution (obtained from Powerful Biology, Wuhan, China), dehydrating with varying acetone and alcohol concentrations, and ultimately embedding in epoxy resin. Uranyl acetate (E. Merck) and lead citrate (Sigma-Aldrich) were employed to stain the ultrathin segments. The observation was conducted employing TEM (JEM1400FLASH, Japan).

Resorption pit assay

BMMs were cultivated on bovine bone segments measuring 6×6 mm and having a thickness of 100 μ m. The cells were then pretreated with 0.5 mmol/L 3-methyladenine (3-MA) or 100 nmol/L rapamycin (RAP) for 1 hour, followed by culture in osteoclastic medium supplemented with 20 μ g/mL YNBY for 5 days. The slices underwent fixation through incubation for 15 to 20 minutes with 4% paraformaldehyde, followed by staining with TRAP solution at 37 °C for 30 minutes. The slices were then allowed to air-dry and were subsequently sputter-coated with a thin gold layer. Next, a field-emission environmental scanning electron microscope (SEM, JSM-IT700HR, Japan) was employed to examine the resorption pits.

Western blotting

The protein samples, consisting of equal amounts (20–30 μ g), were subjected to SDS-polyacrylamide gel electrophoresis and then transferred onto PVDF membranes (Bio-Rad, USA).

The membranes were blocked utilising a 5% (wt/vol) solution of nonfat milk that was diluted at room temperature in TBST (0.05% Tween 20) for 2 hours. Next, the blots were subjected to probing with primary antibodies, which were maintained for the duration of the night at 4 °C. Subsequently, a 2-hour incubation period was conducted with the corresponding peroxidase-coupled secondary antibodies. The ECL detection system (Beyotime, China) was utilised for identifying the immunoreactive protein bands. The quantification of data was conducted utilising the ImageJ programs.

Statistical analysis

The investigation's trials were conducted in 3 separate experiments at a minimum. The outcomes are presented as the means $\pm$ standard deviation (SD). The statistical analysis involved the utilisation of Student t test to perform comparisons between the 2 groups. * $P < .05$ and ** $P < .01$ show significant variations, and NS indicates that there is no significant difference.

Results

YNBY prevented alveolar bone loss in a rat model of ligation-induced periodontitis in vivo

In order to discover the contribution of YNBY in periodontitis, a conventional periodontitis model, specifically ligation-induced periodontitis, was utilised. The μ -CT images demonstrated a significant rise in the CEJ–ABC distance, as detected at 3 distinct points on the buccal aspects of the second molar, in the ligation group in contrast to the control group. Nevertheless, alveolar bone absorption was effectively inhibited at 2, 4, and 6 weeks after YNBY treatment (Figure 1A–F; $P < .05$). Furthermore, the histomorphometric evaluations of segments of tissue stained with HE and TRAP, which encompassed the measurement of the distance between the ABC and CEJ and the quantification of TRAP-positive OCs, produced outcomes that were comparable to those obtained through CT analysis. After YNBY treatment, the CEJ–ABC distance was decreased and the differentiation of OCs was inhibited (Figure 1G–I; $P < .05$).

YNBY inhibited OC differentiation

We first carried out a CCK-8 assay to evaluate the cytotoxic impacts of YNBY on BMMs. The outcomes exhibited that cell viability was not significantly changed by YNBY at concentrations lower than 20 μ g/mL (Figure 2A; $P < .05$). As demonstrated in Figure 2B and C ($P < .05$), the number of TRAP + OCs that included 3 or more nuclei and stained rose red peaked 5 days postculturing. To explore the YNBY impacts on OC differentiation caused by RANKL, BMMs were subjected to various concentrations of YNBY (5, 10, or 20 μ g/mL) or DMSO. Large, mature OCs with multiple nuclei were detected in both the groups of RANKL and DMSO. In contrast, the administration of YNBY exhibited significant dose-dependent inhibition of mature OC development. Additionally, the outcomes of F-actin staining indicated that YNBY effectively suppressed the

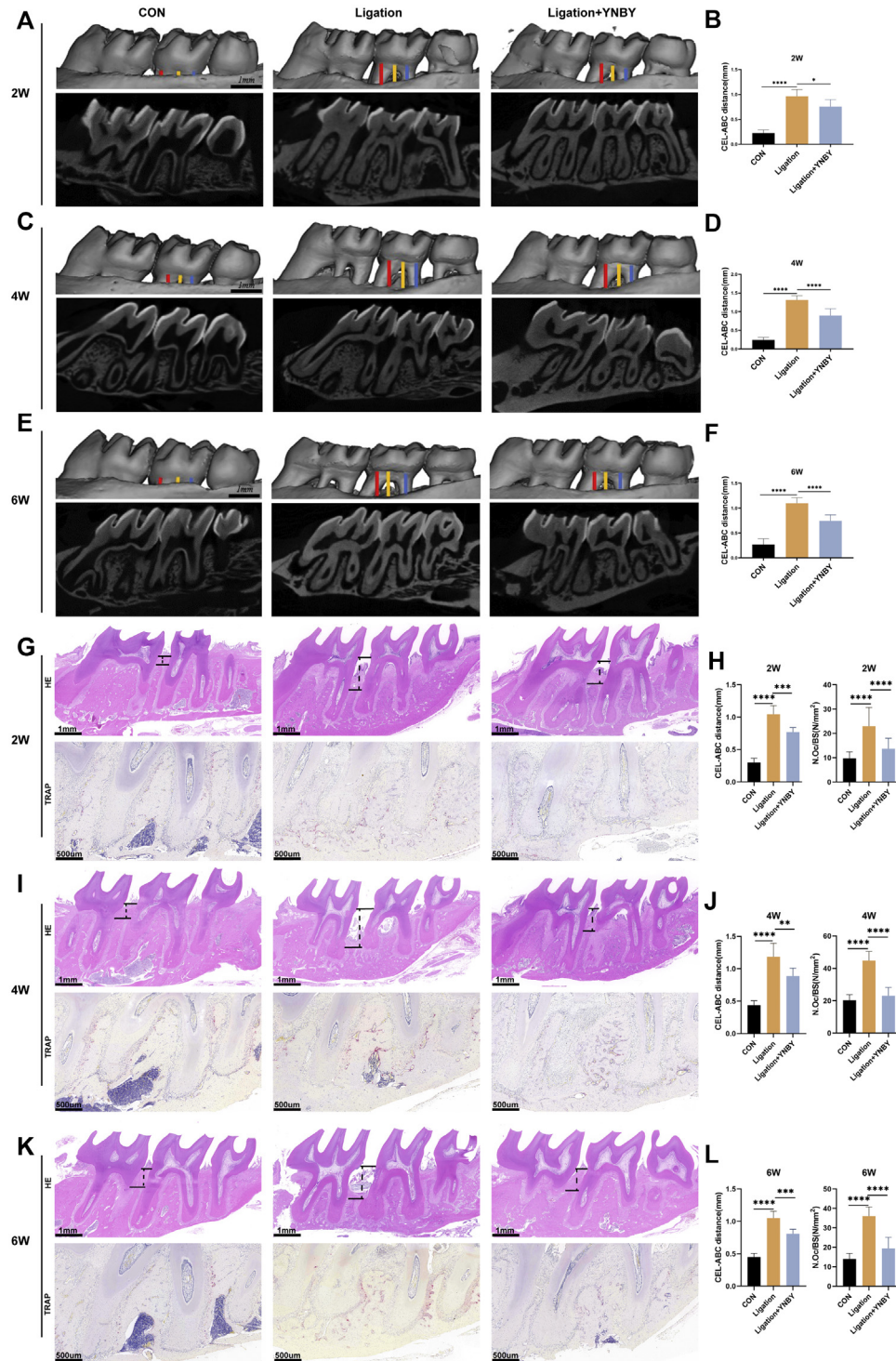


Fig. 1 – Yunnan Baiyao (YNBY) prevented alveolar bone loss and osteoclast differentiation in a ligation-induced periodontitis rat model in vivo. A, C, E, Representative 3-dimensional microcomputed tomography scanning images (upper panels) and reconstructed sections (lower panels) along the longitudinal direction of the maxillae. B, D, F, The distance from the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) in mm was analysed. G, I, K, Representative images of hematoxylin and eosin–stained and tartrate-resistant acid phosphatase (TRAP)–stained paraffin sections. H, J, L, The distance from the CEJ to ABC in mm and the number of alveolar bone surfaces covered by TRAP-positive osteoclasts (N.Oc/BS/mm²) were determined by histomorphologic analysis. The values are expressed as the mean $\pm$ standard deviation. * $P < .05$. ** $P < .01$.

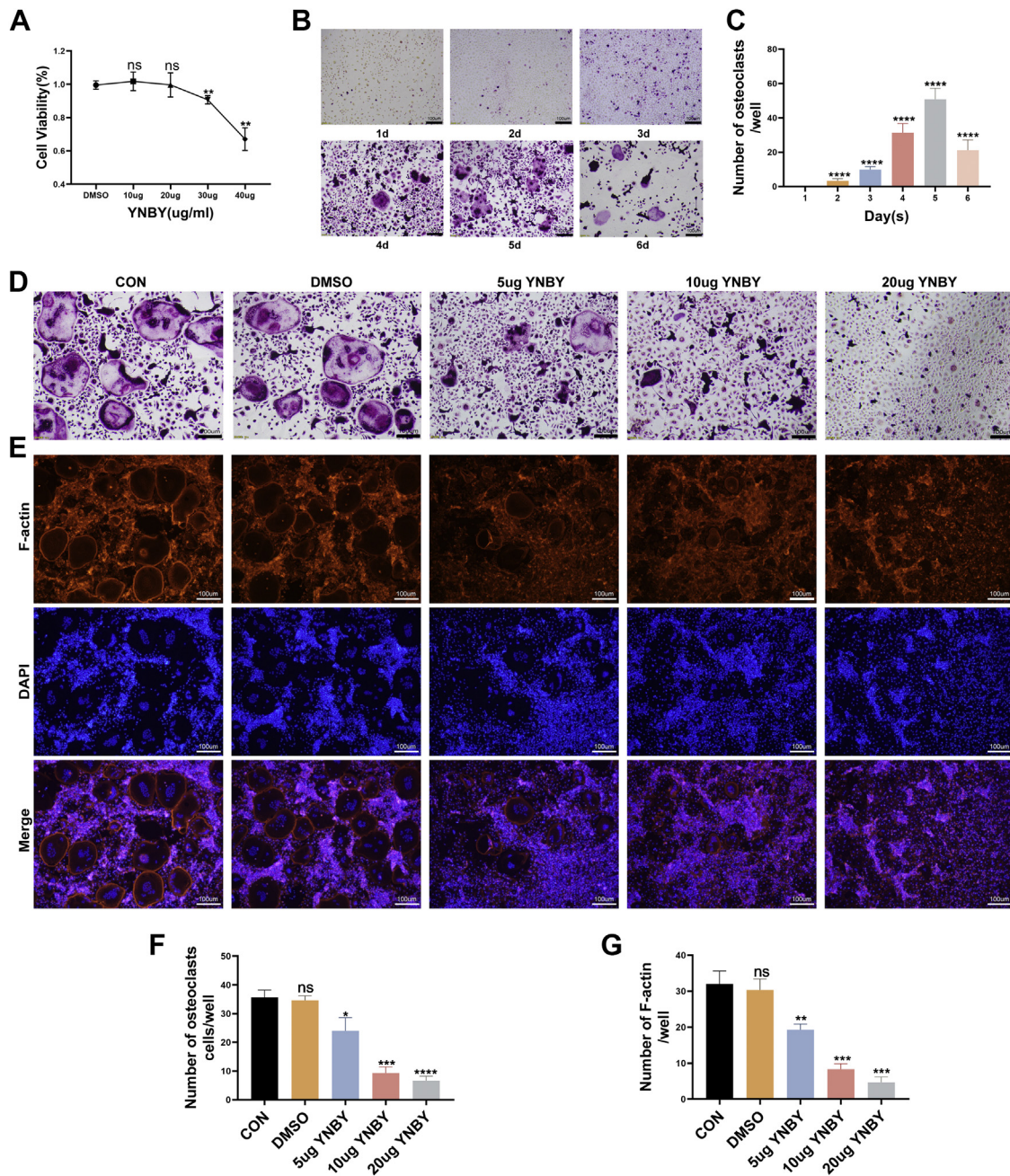


Fig. 2–Yunnan Baiyao (YNBY) inhibited RANKL-induced osteoclastogenesis and the autophagic flux. **A**, Bone marrow–derived macrophages were treated with 25 ng/mL M-CSF and the indicated concentrations of YNBY (10, 20, 30, or 40 $\mu\text{g/mL}$) or dimethylsulfoxide (DMSO), and then cell viability was analysed using a cell counting kit (CCK)–8 assay. **B**, **C**, Cells were stained with TRAP (magnification, 100 $\times$, scale bar = 100 μm) and observed under an inverted phase contrast microscope. The numbers of tartrate-resistant acid phosphatase (TRAP) + multinucleated cells were counted (magnification, 100 $\times$). **D**–**G**, TRAP-positive multinucleated osteoclast numbers and F-actin ring formation were inhibited by YNBY in a dose-dependent manner (5, 10, or 20 $\mu\text{g/mL}$, and DMSO). The results are expressed as the mean $\pm$ SD. * $P < .05$. ** $P < .01$ vs the control.

RANKL-induced development of the F-actin ring in OCs (Figure 2D–G; $P < .05$).

Role of autophagy in YNBY-mediated suppression of OC differentiation

To evaluate the RANKL impacts on the differentiation of OC, GFP-RFP-LC3 fluorescence staining revealed that

RANKL increased the formation of autophagolysosomes, whilst excluding the effect of solvent DMSO on autophagic flux (Figure 3A and B; $P < .05$). Next, to explore whether autophagy was related to the YNBY-mediated inhibition of osteoclastogenesis, the specific autophagy suppressor 3-MA (1 mmol/L) and the autophagy inducer RAP (100 nmol/L) were added to the model of RANKL-induced OC differentiation. The TRAP staining results suggested that 3-MA

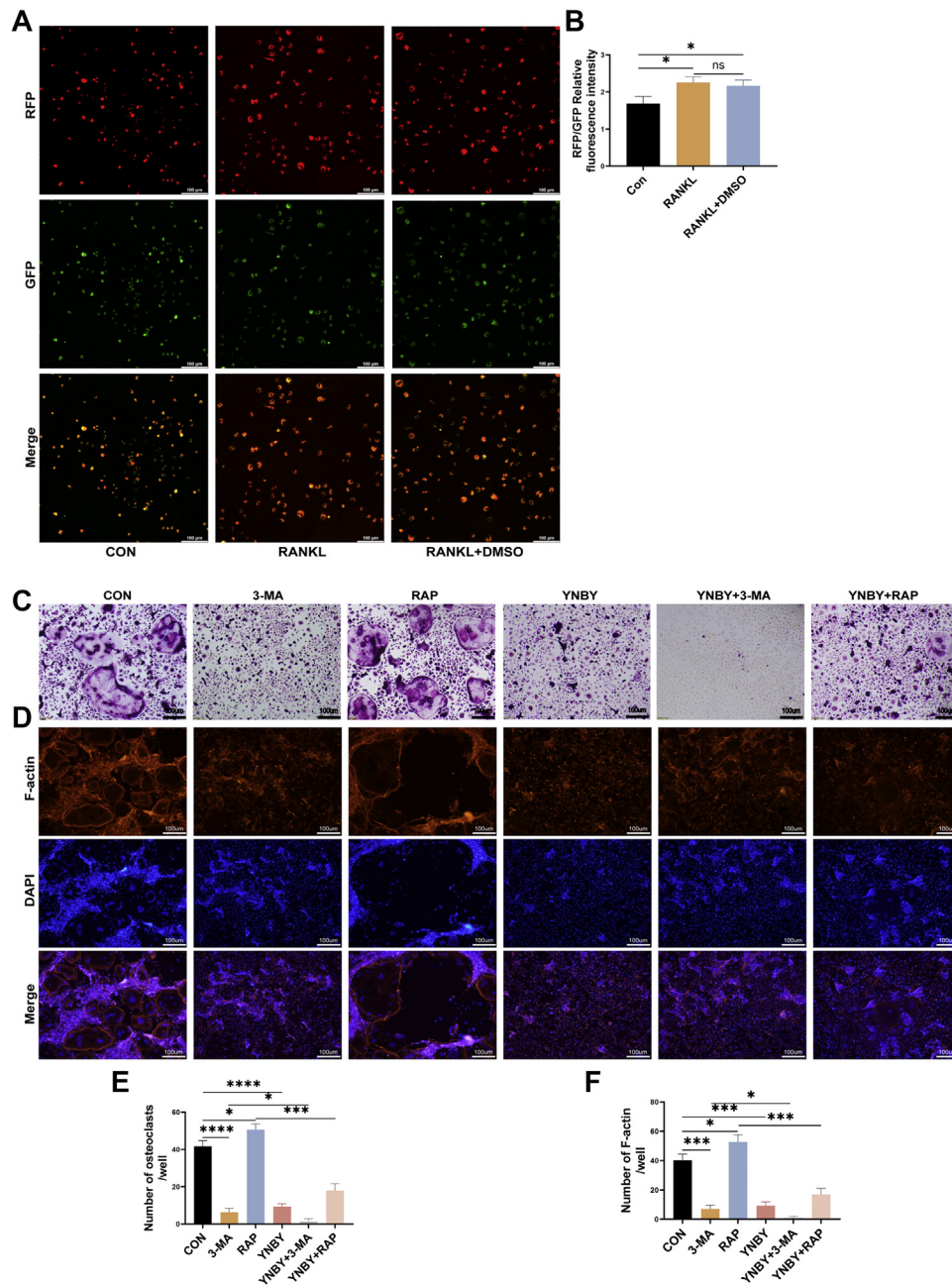


Fig. 3 – Role of autophagy in the Yunnan Baiyao (YNBY)–mediated inhibition of osteoclast differentiation. **A, B**, GFP-RFP-LC3 fluorescence staining revealed that RANKL increased the formation of autophagolysosomes, whilst excluding the effect of solvent dimethylsulfoxide on autophagic flux. **C–F**, YNBY prevented osteoclast differentiation and F-actin ring formation by inhibiting autophagy. * $P < .05$. ** $P < .01$.

inhibited OC differentiation, but RAP facilitated OC differentiation. Then, YNBY + 3-MA significantly inhibited OC differentiation, but YNBY + RAP reduced the effect of RAP on promoting OC differentiation. F-actin staining suggested that 3-MA suppressed the development of the F-actin ring induced by RANKL in OCs, but RAP promoted this process. Meanwhile, YNBY + 3-MA significantly inhibited F-actin ring formation, but the YNBY + RAP

combination reduced the RAP impact on promoting F-actin ring formation (Figure 3C–F; $P < .05$).

Effect of YNBY on the autophagic flux during OC differentiation

The LysoTracker Red staining and TEM results (Figure 4A–C) were generally consistent with the results described above.

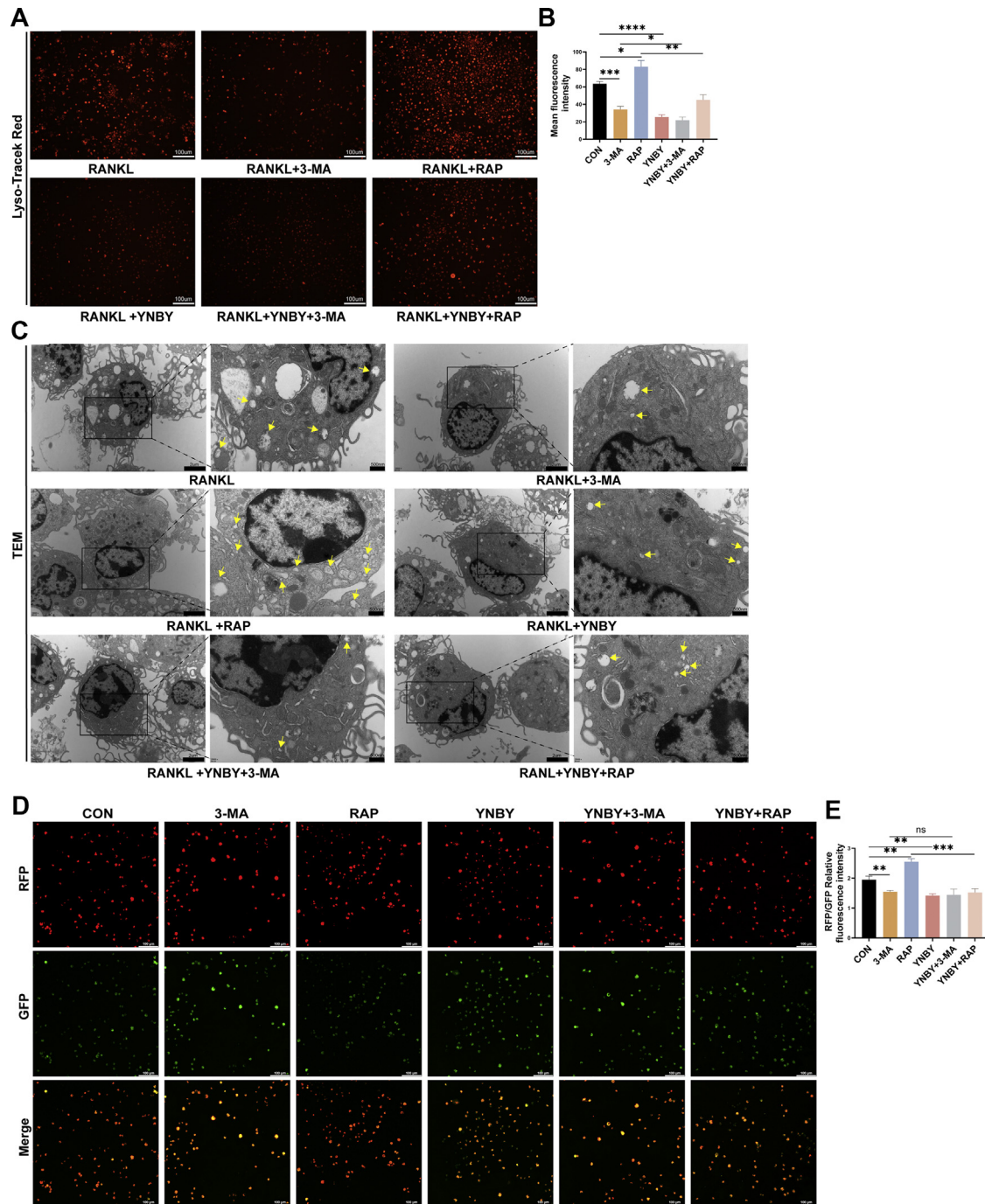


Fig. 4—Effect of Yunnan Baiyao (YNBY) on the autophagic flux during osteoclast differentiation. **A, B**, Bone marrow–derived macrophage (BMM) cells were treated with 20 $\mu\text{g/mL}$ YNBY, 1 mmol/L 3-methyladenine (3-MA), or 100 nmol/L rapamycin (RAP) for 6 hours, and then cells were stained with LysoTracker Red and monitored using confocal immunofluorescence microscopy (scale bar = 100 μm). **C**, Transmission electron microscopy images of BMMs; left panels (magnification, 10,000 $\times$, scale bar = 2 μm) and right panels (magnification, 25,000 $\times$, scale bar = 500 nm). Autophagosomes are indicated by yellow arrows. **D, E**, After infecting BMMs with GFP-RFP-LC3 tandem fluorescent protein adenovirus, the cells were incubated with osteoclast medium supplemented with 20 $\mu\text{g/mL}$ YNBY, 1 mmol/L 3-MA, or 100 nmol/L RAP for 6 hours. Then, fluorescence was observed with a confocal microscope, and quantitative analysis was performed. Scale bar = 100 μm . The data are presented as mean $\pm$ SDs of 3 independent experiments (* $P < .05$, ** $P < .01$). All the experiments were carried out independently at least 3 times (* $P < .05$, ** $P < .01$; bar = 100 μm).

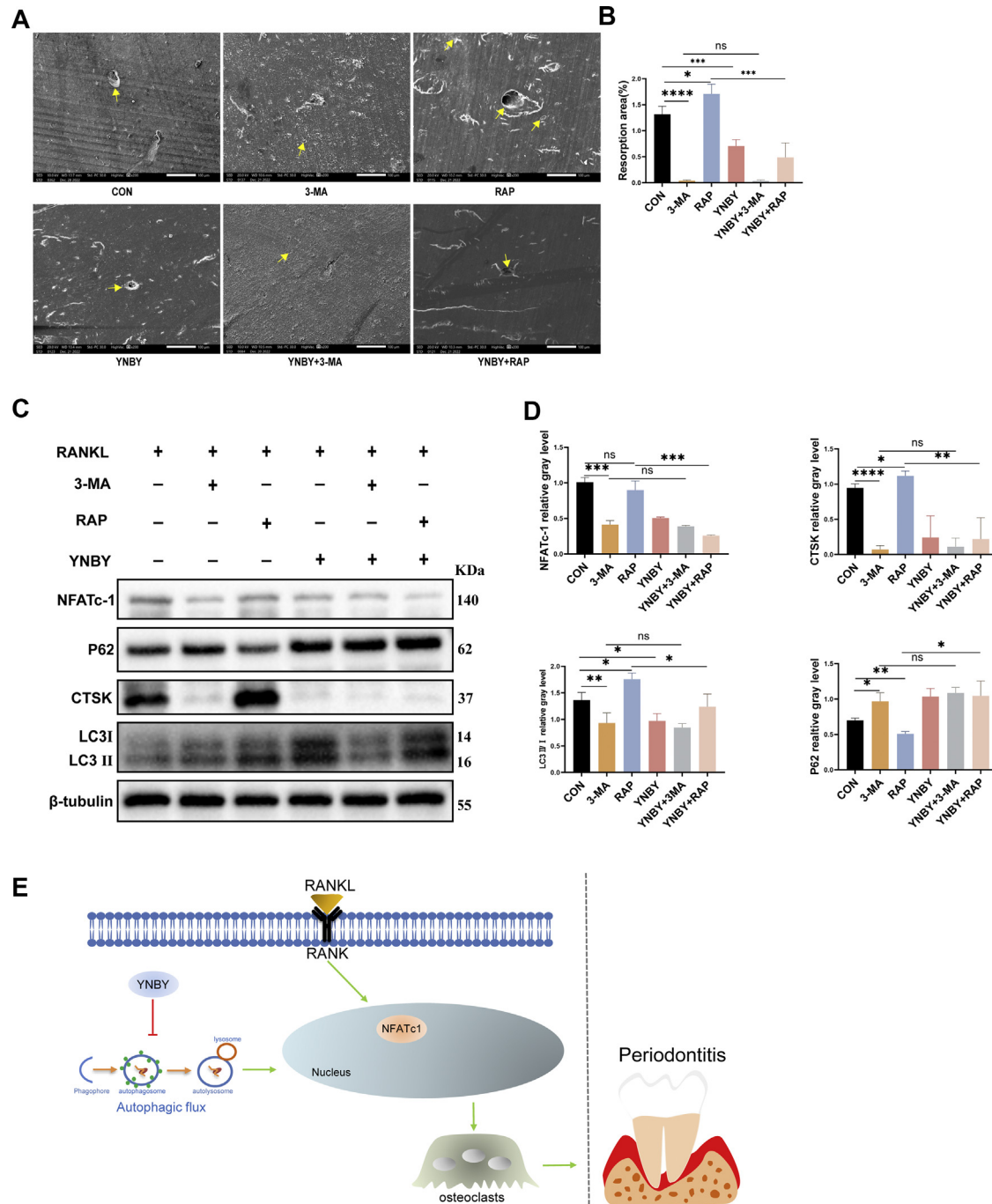


Fig. 5 – Yunnan Baiyao (YNBY) inhibited bone resorption and the expression of osteoclast-related proteins and autophagy-related proteins. A, B, Bone marrow–derived macrophages (BMMs) were seeded on bovine bone slices and allowed to adhere to the surface. BMMs were pretreated with 1 mmol/L 3-methyladenine (3-MA) or 100 nmol/L rapamycin (RAP) for 1 hour and then cultured with osteoclast medium for 5 days. Representative scanning electron microscopy images of bone resorption pits are shown. C, D, BMMs were pretreated with 1 mmol/L 3-MA or 100 nmol/L RAP for 1 hour and then cultured with RANKL for 6 hours. The total proteins were extracted to measure the levels of P62 and LC3. The total proteins were extracted on the 5th day, and western blotting was performed to examine the expression of the osteoclast-related proteins NFATc-1 and CTSK. E, Schematic model of the hypothesized mechanism by which YNBY inhibits osteoclast differentiation. Densitometric analysis of an immunoblot from 3 independent experiments. * $P < .05$. ** $P < .01$.

YNBY significantly inhibited the development of lysosomes and autophagosomes, and the suppressive impact of YNBY on autophagy was reduced after the induction of autophagy. GFP-RFP-LC3 fluorescence staining revealed that 3-MA

significantly inhibited autophagosome development in contrast to the control group, but RAP increased autophagolysosome formation. Furthermore, YNBY + 3-MA significantly inhibited the formation of autophagolysosomes, but the

YNBY + RAP combination inhibited the effect of RAP on promoting autophagosome formation (Figure 4D and E; $P < .05$).

YNBY inhibited bone resorption and the expression of proteins connected to OC and autophagy

Since the differentiation of OCs and the formation of F-actin rings were suppressed by YNBY, we subsequently examined whether YNBY could suppress OC bone resorption. Bone resorption was investigated by scanning electron microscopy after the cells in every group were subjected to the same induction methods. The outcomes exhibited that bone resorption was significantly diminished in the 3-MA group in contrast to the control group, and bone resorption in the group of RAP was significantly increased. YNBY significantly reduced bone resorption after treatment. In addition, the bone resorption of the 3-MA pretreatment + YNBY treatment group was inhibited in contrast to that of the 3-MA single treatment group, and the RAP impact on promoting bone resorption could be significantly inhibited by YNBY treatment (Figure 5A and B). Next, we further verified the YNBY effect on the expression of proteins connected to OCs and autophagy. As presented in Figure 5C and D, 3-MA suppressed the protein expression levels of NFATc-1 and CTSK, but RAP increased their levels, whilst the YNBY + RAP combination inhibited the RAP effect on promoting these proteins' expression. Furthermore, the LC3II/I levels were significantly reduced ($P < .01$) and the P62 protein levels were significantly increased ($P < .01$) in the 3-MA group. The LC3II/I and P62 protein levels were significantly decreased ($P < .01$) in the RAP group. Both YNBY + 3-MA and YNBY + RAP groups exhibited decreased LC3II/I levels and increased P62 protein levels. These results proved that induction of autophagy promotes differentiation of OCs and bone resorption, and YNBY may suppress OC differentiation and bone resorption by inhibiting the autophagic flux (Figure 5E).

Discussion

Periodontitis is characterised by the activation of local inflammatory reactions in periodontal tissues, which are induced by microbes and toxic products found in bacterial plaques. This process is mediated by the synthesis of inflammatory mediators on a large scale, which can stimulate OC activity and aggravate alveolar bone resorption.^{12,13}

OCs are the unique cell type accountable for bone resorption within the human body, mainly differentiated from mononuclear macrophages, and OCs have a significant function in bone metabolism.^{14,15} The bone resorption process is performed by OCs, which exhibit polarity in their secretion of protons and proteolytic enzymes within a sealed bone region known as the resorption lacunae.¹⁶

Autophagy is a phenomenon by which cells digest and decompose substrates, such as organelles and biomacromolecules, in secondary lysosomes; this process is regulated by proteins that are encoded by autophagy-related genes, and autophagy is strictly connected to the OCs' differentiation, apoptosis, and function.^{17,18} Furthermore, certain academic researchers have discovered that autophagy serves crucial functions in the processes of inflammation and bone

resorption in the context of periodontitis. The experimental models of periodontitis that exhibit elevated levels of autophagy may experience a decrease in inflammation, osteoclastogenesis, and bone resorption upon pharmacologic suppression of autophagy through the use of 3-MA or chloroquine (CQ).^{19,20} Recently, some investigations have shown that autophagy is also strictly associated with periodontitis incidence and progression; some researchers discovered that in periodontitis, in periodontal ligament stem cells, the autophagy level was greater than that in normal tissues, as indicated by increased protein expression of LC3, Beclin-1, ATG7, and ATG12 and significant increases in the number of autophagosomes.²¹ In a nonprimate periodontitis model, the early autophagy gene ULK and the late autophagy gene ATG12 were significantly upregulated in the gingival tissues of the periodontitis side (1–3 months) according to microarray screening.²²

Based on a comprehensive literature review and previous research conducted by our research team, it was observed that the YNBY exhibited favourable anti-osteoclastogenic properties. To further investigate the precise impact of YNBY on periodontitis, a series of control experiments were conducted. The anti-osteoclastogenic effect of YNBY was successfully demonstrated in an experimental periodontitis rat model. However, the underlying mechanism responsible for this effect remains unclear. Consequently, a series of in vitro experiments were subsequently conducted to examine the impact of YNBY on the differentiation of OC precursors and the functionality of mature OCs. The present study has demonstrated that YNBY treatment leads to a decrease in the expression of autophagy-related genes as well as a reduction in the numbers of autophagosomes and autolysosomes and YNBY suppresses the differentiation of OCs and bone resorption during periodontitis by blocking autophagy. Nevertheless, the precise mechanism by which YNBY affects autophagy-related genes remains uncertain and requires additional confirmation. Nonetheless, YNBY holds potential as a novel therapeutic approach for modulating autophagy in the treatment of metabolic diseases, including periodontitis.

Conclusions

Our outcomes indicate that autophagic flux level is positively correlated with OC differentiation. YNBY inhibits the differentiation of OCs and bone resorption in vitro and in vivo, and YNBY inhibits the autophagic flux during OC differentiation. These findings further demonstrated that YNBY may inhibit OC differentiation and activity by downregulating the autophagic flux and reducing alveolar bone loss in an investigational rat model of periodontitis. These findings reveal a novel mechanism by which YNBY may function in the treatment of periodontitis.

Conflict of interest

None disclosed.

Acknowledgements

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Author contributions

Hongbing He and Xiaobin Ren conceived the project. Yanjie Li and Wang Liu designed and performed experiments. Yanjie Li and Wang Liu have contributed equally to this work. Ruoyu Zhao and Yuanyuan An evaluated and interpreted the information. Hongbing He and Xiaobin Ren were accountable for drafting the manuscript. The final manuscript was reviewed and authorised by all authors.

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