

Inhibitory Effect of *Allium cepa* L.-Derived Extracellular Vesicles Loaded with Celecoxib on Osteoclast Differentiation in Periodontitis

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Purpose: Alveolar bone resorption in periodontitis is the primary clinical cause of tooth loss. This study aimed to investigate the inhibitory effect of *Allium cepa* L.-derived extracellular vesicles (AC-EVs) loaded with celecoxib (ACEV@CEL) on alveolar bone resorption.

Methods: AC-EVs were isolated using gradient centrifugation in combination with ultracentrifugation, after which celecoxib was incorporated via ultrasonication to generate ACEV@CEL. Subsequently, a rat periodontitis model was established, and local administration was performed to systematically evaluate the biocompatibility and therapeutic effects on alveolar bone resorption. In vitro, after determining the optimal administration dose for each treatment group, we confirmed that RAW264.7 cells were able to internalize AC-EVs and ACEV@CEL. In the established in vitro periodontitis model, ACEV@CEL significantly inhibited osteoclast differentiation, and this inhibitory effect was stronger than that of either AC-EVs or celecoxib alone.

Results: In vivo, ACEV@CEL exhibited good biocompatibility and effectively suppressed alveolar bone resorption in rats with periodontitis. In vitro, ACEV@CEL was internalized by RAW264.7 cells and inhibited their differentiation into osteoclasts.

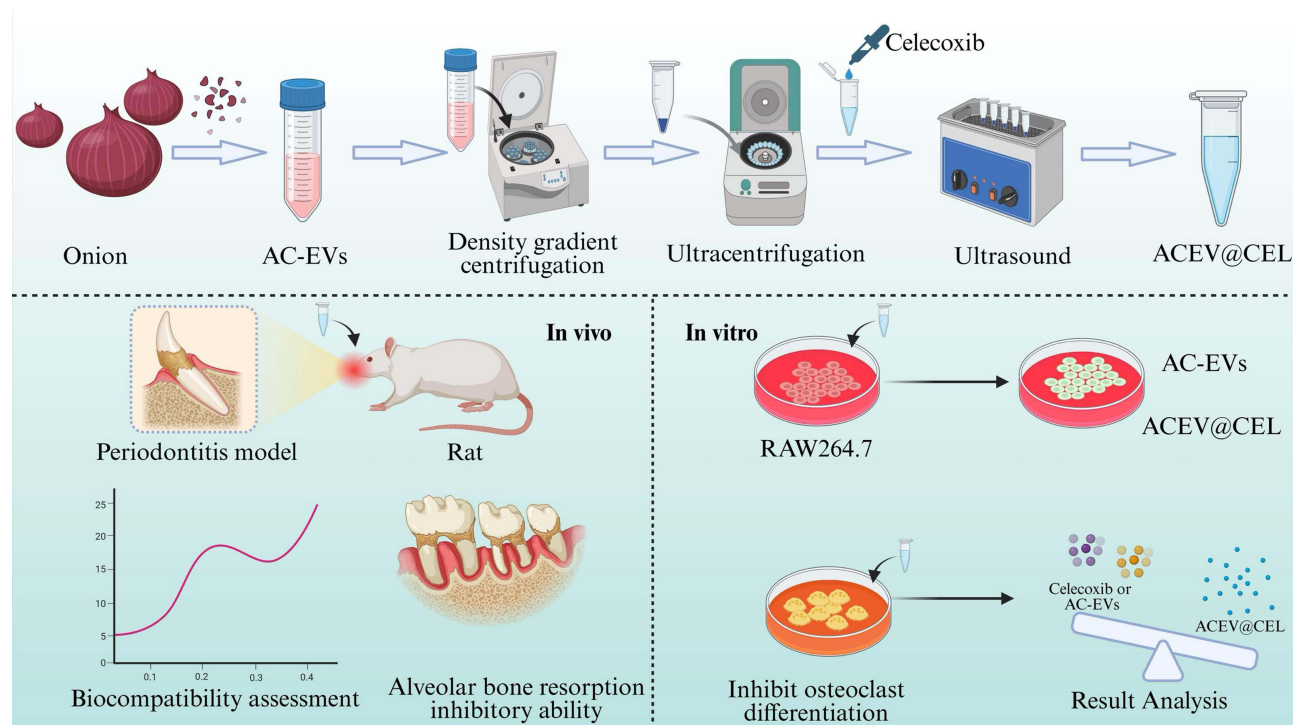
Conclusion: ACEV@CEL is able to suppress osteoclast differentiation under periodontitis conditions, while demonstrating favorable biocompatibility and safety, suggesting its potential as a therapeutic agent for periodontitis and warranting further long-term investigation.

Keywords: periodontitis, plant-derived extracellular vesicles, osteoclast

Introduction

Periodontitis is a chronic oral disease characterized by gingival bleeding, periodontal pocket formation, alveolar bone resorption, and attachment loss, and represents one of the leading causes of tooth loss worldwide, affecting approximately 40–70% of the global population.¹ It is now widely accepted that periodontitis is a complex inflammatory disease of periodontal tissues primarily driven by *Porphyromonas gingivalis* (*P. gingivalis*), in conjunction with other pathogenic bacteria such as *Tannerella forsythia* and *Aggregatibacter actinomycetemcomitans*,² which collectively contribute to the initiation and progression of periodontal inflammation.³ The pathogenesis of periodontitis involves intricate bacteria–host interactions and is regulated by multiple factors, including genetic polymorphisms,⁴ cytokine expression (in supragingival, subgingival, and salivary environments),⁵ viral infections,⁶ and oxidative stress,⁷ ultimately leading to the destruction of periodontal supporting tissues. Under physiological conditions, a dynamic balance between alveolar bone resorption and formation is maintained in healthy periodontal tissues, thereby preventing bone loss. However, local microbial dysbiosis in periodontal tissues triggers host immune responses, resulting in a marked increase in pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α),⁸ interleukin-1 β (IL-1 β), and interleukin-6 (IL-6), which induce osteocyte apoptosis. During osteocyte apoptosis, cytokines such as IL-6 promote the release of receptor activator of

Graphical Abstract



nuclear factor κ B ligand (RANKL). RANKL binds to its receptor RANK on monocyte/macrophage lineage cells, leading to the recruitment of tumor necrosis factor receptor-associated factor 6 (TRAF6) in the cytoplasm and subsequent activation of classical signaling pathways, including PI3K/Akt, MAPK, and NF- κ B. Activation of these pathways promotes osteoclast differentiation and enhances bone resorption through upregulation of osteoclast-related proteins such as cellular Fos (c-Fos), Nuclear Factor of Activated T Cells 1 (NFATc1), and cathepsin K (CTSK).⁹ Moreover, apoptotic osteocytes and their apoptotic bodies can directly regulate osteoclastogenesis and bone remodeling by secreting RANKL. These apoptotic bodies interact with specific markers on osteoclast precursor cells, thereby promoting TNF- α gene expression and inducing osteoclast formation.¹⁰ Furthermore, osteocyte-derived apoptotic bodies may undergo secondary apoptosis, leading to the release of various pro-inflammatory cytokines, which subsequently activate immune cells to upregulate RANKL expression and further promote osteoclastogenesis.¹¹

Plant-derived extracellular vesicles (PDEVs) were first isolated from sunflower seeds by Regente et al, who adopted extraction methods similar to those used for mammalian-derived extracellular vesicles (MDEVs), involving gradient centrifugation followed by ultracentrifugation.¹² With continuous methodological optimization, commonly used extraction techniques now include juice extraction followed by ultrafiltration, gradient centrifugation, ultracentrifugation, and sucrose density gradient centrifugation.¹³ Most PDEVs possess a simple lipid bilayer structure.¹⁴ PDEVs can be isolated from a variety of fruits and vegetables, including ginger,¹⁵ grapefruit,¹⁶ and carrot,¹⁷ as well as from woody plants and herbal medicines such as ginseng¹⁸ and green tea.¹⁹ Structurally, PDEVs contain lipids (without cholesterol²⁰), proteins (with lower diversity and abundance compared to MDEVs²¹), and miRNAs, resembling MDEVs. Functionally, PDEVs exhibit multiple biological activities, including anti-inflammatory,²² antioxidant,²³ and anti-tumor effects.²⁴ Moreover, their abundant sources, ease of extraction, low toxicity, low immunogenicity, and high bioavailability distinguish them from conventional therapeutics, highlighting their broad application potential. Compared with single bioactive compounds that are rapidly metabolized in vivo, PDEVs can encapsulate plant-derived active components within their

phospholipid membranes, protecting them from degradation and thereby facilitating the preservation of their biological functions.

Onion (*Allium cepa* L.) belongs to the genus *Allium* and is widely used not only as a culinary ingredient but also for medicinal purposes. Due to its excellent storage stability and transport durability, onions have a broader trade distribution compared with most vegetables. Onions contain approximately 90% water and are rich in dietary fiber and carbohydrates. In terms of micronutrient composition, onions are characterized by relatively low sodium content and high levels of vitamin B6, folate, calcium, magnesium, phosphorus, and potassium.²⁵ The diverse bioactive components present in onions are central to their biological functions. For example, quercetin strongly inhibits the release of prostaglandin E2, leukotrienes, and histamine,²⁶ thereby conferring potent anti-inflammatory effects. In addition, thiosulfates can inhibit the arachidonic acid metabolic pathway, suppress the release of pro-inflammatory cytokines, and interfere with NF- κ B transcription.²⁷ Lee et al demonstrated that onion peel extract inhibits IL-1 β and IL-6 expression as well as JAK-STAT pathway activation in an LPS-induced inflammatory model.²⁸ Furthermore, onions exhibit preventive effects against various cancers. Bioactive compounds such as quercetin, organosulfur compounds, kaempferol derivatives, and organoselenium possess strong anticancer activities.²⁹ For instance, Veiga et al reported that quercetin extracted from onions exerted significant cytotoxic effects on adrenocortical carcinoma cell lines H295R and SW-13, with effective concentrations lower than that of the clinically used drug mitotane,³⁰ thereby providing a novel direction for clinical treatment. Beyond these functions, Tang et al demonstrated that aqueous onion extracts inhibit osteoclast differentiation from mouse bone marrow cells and RAW264.7 macrophages, while also suppressing osteoclastic resorptive activity.³¹ Zhang et al further showed that onion-derived flavonoids promote proliferation of human osteoblast-like MG-63 cells while inhibiting osteoclast differentiation in RAW264.7 cells.³² Given that osteoclast formation is largely driven by inflammatory cytokines, and that PDEVs often carry multiple bioactive components derived from their source plants, *Allium cepa* L.-derived extracellular vesicles (AC-EVs) may exert inhibitory effects on osteoclast differentiation.

Celecoxib is a selective cyclooxygenase-2 (COX-2) inhibitor and a second-generation nonsteroidal anti-inflammatory drug (NSAID), widely used for the treatment of inflammatory diseases and pain. Compared with traditional NSAIDs, celecoxib exhibits higher COX-2 selectivity, thereby reducing gastrointestinal adverse effects while effectively suppressing inflammatory responses. Studies have shown that celecoxib not only exerts anti-inflammatory effects but also regulates bone metabolism. For example, in lipopolysaccharide (LPS)-induced apical periodontitis, celecoxib significantly inhibits osteoclast formation in periapical tissues, thereby reducing bone resorption.³³ In addition, in a *Staphylococcus aureus*-induced mouse osteomyelitis model, celecoxib treatment effectively restores bone volume fraction and trabecular number while markedly reducing osteoclast numbers compared with the osteomyelitis group, demonstrating its therapeutic efficacy in bone destruction.³⁴ However, celecoxib is classified as a Biopharmaceutics Classification System (BCS) class II drug, characterized by high lipophilicity but extremely low aqueous solubility.³⁵ Therefore, in aqueous environments such as gingival crevicular fluid, its poor solubility results in slow dissolution and difficulty in rapidly achieving effective local therapeutic concentrations. Moreover, the continuous flow of gingival crevicular fluid within periodontal pockets can rapidly clear the drug, thereby reducing its local bioavailability. As a natural drug delivery carrier, extracellular vesicles (EVs) possess a lipid bilayer structure capable of encapsulating hydrophobic drugs, enhancing their solubility and facilitating cellular uptake,³⁶ while also conferring intrinsic biological functions.

Given the current limitations of therapeutic strategies directly targeting alveolar bone resorption in periodontitis, this study aimed to load celecoxib into AC-EVs to generate ACEV@CEL, and to investigate whether ACEV@CEL exerts a therapeutic effect on alveolar bone resorption in a rat model of periodontitis, as well as its effect on the differentiation of RAW264.7 cells into osteoclasts in vitro. We hypothesized that ACEV@CEL would reduce alveolar bone loss in rats with periodontitis and inhibit the differentiation of RAW264.7 cells into osteoclasts. Conversely, if ACEV@CEL exerted no significant effect, the degree of alveolar bone resorption in rats and the osteoclast differentiation capacity of RAW264.7 cells would not differ significantly from those observed in groups treated with AC-EVs or celecoxib alone, or may even show no inhibitory effect on osteoclast differentiation. If the anticipated outcomes are achieved, this study

may provide a novel therapeutic strategy and new insights for the treatment of alveolar bone resorption in patients with periodontitis.

Materials and Methods

Materials and Instruments

RAW264.7 cells were obtained from Pricella (China). Dulbecco's Modified Eagle Medium (DMEM), penicillin-streptomycin, and phosphate-buffered saline (PBS) were supplied by Gibco (USA). Fetal bovine serum (FBS) was sourced from Clark (USA). Celecoxib was acquired from Solarbio (China), while isoflurane was provided by RWD (China). Recombinant Murine Macrophage colony-stimulating factor (M-CSF) and Recombinant Murine sRANKL were obtained from PeproTech (USA). *P. gingivalis* LPS was procured from InvivoGen (France). Reagents including 4% paraformaldehyde, RIPA lysis buffer, phenylmethylsulfonyl fluoride (PMSF), BCA protein assay kit, hematoxylin and eosin (H&E) staining kit, DAPI, and phalloidin were all obtained from Beyotime Biotechnology (China). 0.22 μm and 0.45 μm filters, as well as polyvinylidene fluoride (PVDF) membranes, were provided by Millipore (USA). Ultrafiltration tubes (100 kDa) were supplied by Amicon (USA). The tartrate-resistant acid phosphatase (TRAP) staining kit was obtained from Servicebio (China). Dil (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) staining dye was purchased from Thermo Fisher (USA). The primary antibodies used in this study included rabbit anti-Cathepsin K antibody (Proteintech, USA), rabbit anti-NFATc1 antibody (Abclonal, China), and rabbit anti-TRAF6 antibody (Abclonal, China), while the secondary antibody (goat anti-rabbit IgG) was provided by Affinity Biosciences (USA).

An ultracentrifuge (Beckman Coulter, USA) was utilized for AC-EVs isolation. Nanoparticle tracking analysis (NTA) was conducted using NanoSight NS300 (Malvern Panalytical, UK). The morphology of AC-EVs and ACEV@CEL was examined by transmission electron microscopy (TEM) (Hitachi, Japan). The rat maxillae were scanned using a micro-computed tomography system (Micro-CT) (Bruker Skyscan, Belgium), followed by three-dimensional reconstruction and analysis using Mimics 21 software. The absorbance was measured using an ELISA microplate reader (Thermo Fisher, USA). The internalization of AC-EVs and ACEV@CEL by RAW264.7 cells was visualized using a laser scanning confocal microscope (Nikon, Japan).

Isolation of AC-EVs

Fresh red onions were washed, peeled, and homogenized in cold PBS. The homogenate was sequentially centrifuged at low speeds to remove cell debris and large particles. The obtained pellet was washed with PBS and further purified by sucrose density gradient ultracentrifugation. Fractions corresponding to the typical density of extracellular vesicles were collected, diluted in PBS, and ultracentrifuged again to obtain purified vesicles, which were finally resuspended in PBS and stored at -80°C for further use. Detailed experimental procedures are provided in Section 1 (Extraction of AC-EVs) of the [Supplementary Materials](#).

Preparation of AC-EV@CEL

Celecoxib was mixed with an equal volume of AC-EVs and placed on ice. The mixture was sonicated using an ultrasonic homogenizer, and after sonication, the suspension was incubated at 37°C to stabilize the extracellular vesicle membranes. Unbound AC-EVs were removed by ultrafiltration, resulting in the preparation of ACEV@CEL. The extraction of AC-EVs and the loading process of AC-EV@CEL are illustrated in the schematic diagram shown in [Figure 1](#). Detailed experimental procedures are provided in Section 2 (Preparation of ACEV@CEL) of the [Supplementary Materials](#).

Measurement of Drug Loading and Encapsulation Efficiency of AC-EVs

Different concentrations of celecoxib solutions were prepared, and the absorbance at 252 nm was measured using a microvolume spectrophotometer to construct a standard curve for celecoxib. Then, the absorbance of ACEV@CEL at 252 nm was measured, and the corresponding celecoxib concentration was calculated using the standard curve equation.

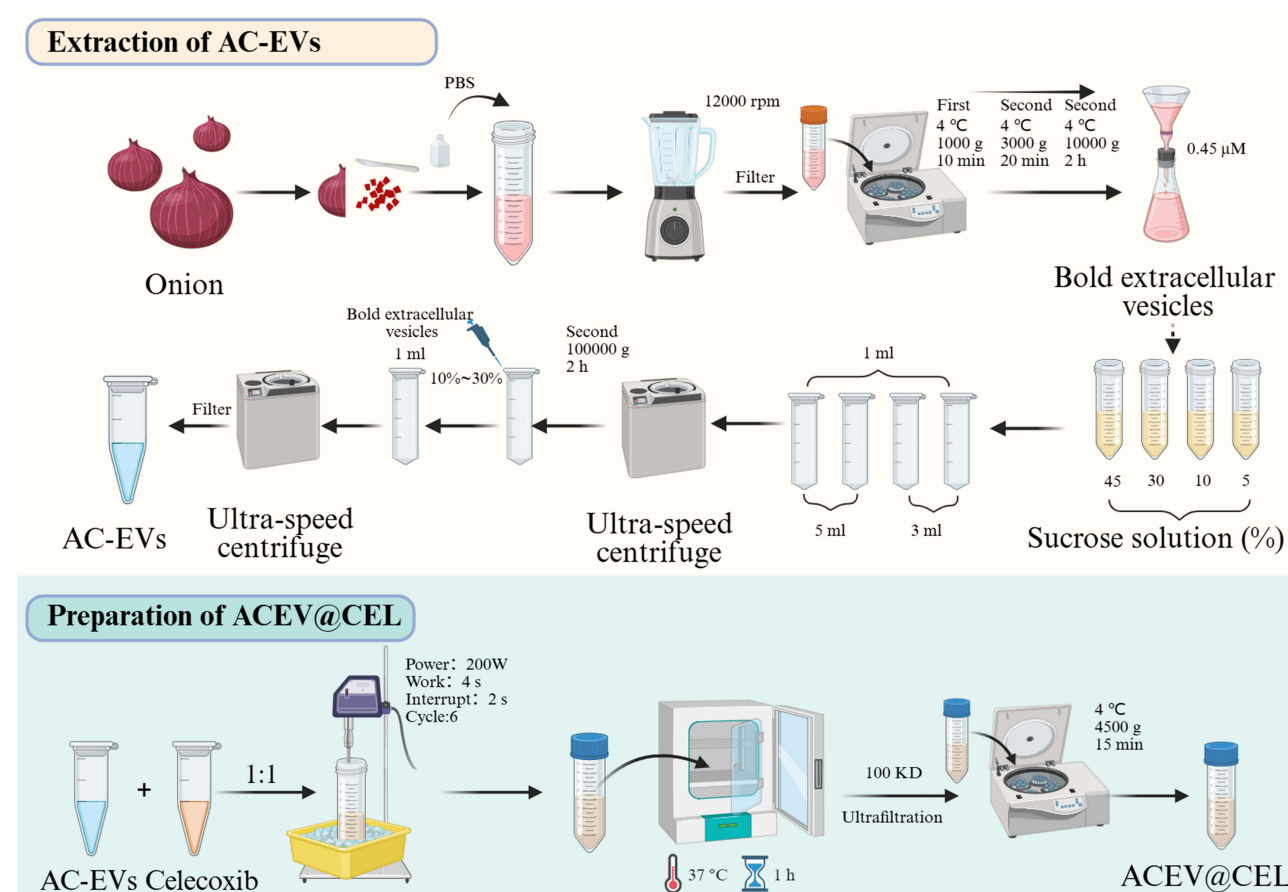


Figure 1 Schematic illustration of the isolation and purification of AC-EVs, and the preparation of ACEV@CEL via ultrasonic co-incubation.

Detailed experimental procedures are provided in Section 3 (Determination of Drug Loading in ACEV@CEL) of the [Supplementary Materials](#).

Characterization of AC-EVs and ACEV@CEL

NTA: Purified AC-EVs and ACEV@CEL were diluted with PBS solution up to 1000 times. The particle size was measured using the NTA system. TEM Observation: Morphological features of EVs were observed using negative staining. Detailed experimental procedures are provided in Section 5 (Characterization of AC-EVs and ACEV@CEL) of the [Supplementary Materials](#).

Establishment and Grouping of the Rat Periodontitis Model

This study included 30 6-week-old SD rats (purchased from Sibeifu, China), weighing 180–200 g. The rats were housed under conditions of 21 ± 1 °C temperature and 65–75% relative humidity with free access to water. After 1 week of adaptive feeding, experiments were initiated. All animal experiments were approved by the Animal Ethics Committee of China Medical University (Ethical approval number: CMU202411).

The 30 rats were randomly divided into five groups (n = 6): the negative control group (CON group), where PBS was injected into the periodontal pockets of the bilateral maxillary first molars every 2 days; the periodontitis group (PD group), where rats were anesthetized with isoflurane, and orthodontic ligature wire was placed around the cervical region of the bilateral maxillary first molars, followed by the application of *P. gingivalis* LPS to induce periodontitis; the AC-EVs group, where AC-EVs were injected into the periodontal pockets every 2 days during periodontitis induction; the CEL group, where celecoxib (CEL) was injected into the periodontal pockets every 2 days during periodontitis induction; and the ACEV@CEL group, where ACEV@CEL was injected into the periodontal pockets every 2 days during

periodontitis induction. After 4 weeks, Rats were euthanized using CO₂ inhalation in accordance with institutional guidelines, and tissues including the heart, liver, spleen, lung, kidney (stored at room temperature in 4% paraformaldehyde), bilateral maxilla (stored at room temperature in 4% paraformaldehyde) were collected. Detailed experimental procedures are provided in Section 6 (Establishment of the Rat Periodontitis Model and Grouping) of the [Supplementary Materials](#).

Biosafety Evaluation

Four weeks after model establishment, rats were euthanized, and tissues including the heart, liver, spleen, lung and kidney were collected to assess whether the vital organs were damaged. Detailed experimental procedures are provided in Section 10 (Biosafety Evaluation) of the [Supplementary Materials](#).

Micro-CT Analysis

Micro-CT was used to scan and reconstruct three-dimensional images of the rats' maxilla. Three-dimensional reconstruction was performed using Mimics 21 software, and corresponding three-dimensional and two-dimensional images were generated. The distance from the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) between the maxillary first and second molars was measured as an indicator of alveolar bone loss. Detailed experimental procedures are provided in Section 7 (Micro-CT Evaluation) of the [Supplementary Materials](#).

Histological Detection

After decalcification in 10% EDTA for 4 weeks, samples were embedded in paraffin and sectioned into 2 µm thick slices using a microtome, followed by storage at room temperature. H&E staining and TRAP staining were subsequently carried out to assess tissue morphology and quantify osteoclasts. Detailed experimental procedures are provided in Section 9 (Histological Staining) of the [Supplementary Materials](#).

Cell Culture

RAW264.7 cells from mouse monocytic leukemia were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin. The cells were maintained at 37 °C in a 5% CO₂ environment. Detailed experimental procedures are provided in Section 11 (Culture of RAW264.7 Cells) of the [Supplementary Materials](#).

Evaluation of the Internalization of AC-EVs and AC-EV@CEL by RAW264.7 Cells

Dil dye was added to AC-EVs and ACEV@CEL and incubated at 37 °C for 30 minutes. Unbound dye was removed by centrifugation, and the concentration was measured before adding the solution to RAW264.7 cells. PBS was used as the control. After incubation and fixation, the cells were stained with phalloidin and DAPI to label the cytoskeleton and nuclei, respectively, and the internalization of EVs by the cells was observed using confocal microscopy. Detailed experimental procedures are provided in Section 12 (Evaluation of the Internalization of AC-EVs and ACEV@CEL by RAW264.7 Cells) of the [Supplementary Materials](#).

CCK-8 Assay for Cell Viability

Different concentrations of AC-EVs, CEL, and ACEV@CEL were co-cultured with RAW264.7 cells (n=3). After incubation, 10 µL of CCK-8 solution was added to each well, followed by a 4-hour incubation. Cell viability in each group was determined by measuring the absorbance at 450 nm using an ELISA microplate reader. Detailed experimental procedures are provided in Section 13 (CCK-8 Assay for Cell Viability) of the [Supplementary Materials](#).

TRAP Staining to Evaluate Osteoclast Differentiation

After overnight plating of RAW264.7 cells, M-CSF and RANKL were added to the different groups. Cells were treated with AC-EVs, Celecoxib, and ACEV@CEL according to the appropriate concentration, while the control group received equal volumes of PBS (n=3). The culture medium was replaced every 2 days until multinucleated osteoclasts were visible under a microscope. The treatment was then stopped, and TRAP staining was performed to count the TRAP-positive

cells in each group. Detailed experimental procedures are provided in Section 14 (TRAP Staining to Evaluate the Effects of Different Treatments on Osteoclast Differentiation) of the [Supplementary Materials](#).

Western Blotting

Experimental grouping of cells: Cells were treated with AC-EVs, Celecoxib, and ACEV@CEL according to the appropriate concentration, while the control group received equal volumes of PBS (n=3).

Protein Extraction from Tissue and Cell Samples: RIPA lysis buffer was added to cell samples, and cells were scraped and collected. After sonication on ice for 10 minutes, the supernatant was collected after centrifugation. Protein concentration was measured, and proteins were denatured at 95 °C and stored at −80 °C. **Western Blotting:** SDS-PAGE gels were loaded at 80 V for 30 minutes, followed by 120 V for 60 minutes. Proteins were transferred from the gel to a polyvinylidene fluoride membrane at 200 mA. The membrane was blocked with 5% milk and incubated with the primary antibody overnight at 4 °C. After incubation with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature, chemiluminescence detection was performed using a TANON 5500 Chemiluminescence Developer (Tanon, China) and chemiluminescent reagents. Protein bands were quantified using Image J(version 1.53, National Institutes of Health, USA). Detailed experimental procedures are provided in Section 15 (Western Blotting) of the [Supplementary Materials](#).

Statistical Analyses

All quantitative data are presented as the mean ± standard deviation (SD). Statistical analyses were conducted using SPSS Statistics 26.0 (IBM Corp., Armonk, NY, USA). For comparisons among multiple groups, one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was applied. At least three biological replicates were included in each group. A *p* value < 0.05 was considered statistically significant.

Results

Identification of AC-EVs and ACEV@CEL

According to the scheme illustrated in [Figure 1](#), AC-EVs were successfully isolated and subsequently loaded with celecoxib. To characterize their particle size distribution and morphological features, NTA was employed to determine vesicle diameter, and TEM was used to visualize their morphology. As shown in [Figure 2](#), NTA results revealed that the particle sizes of AC-EVs and ACEV@CEL were predominantly distributed within the ranges of 94–213 nm ([Figure 2a](#))

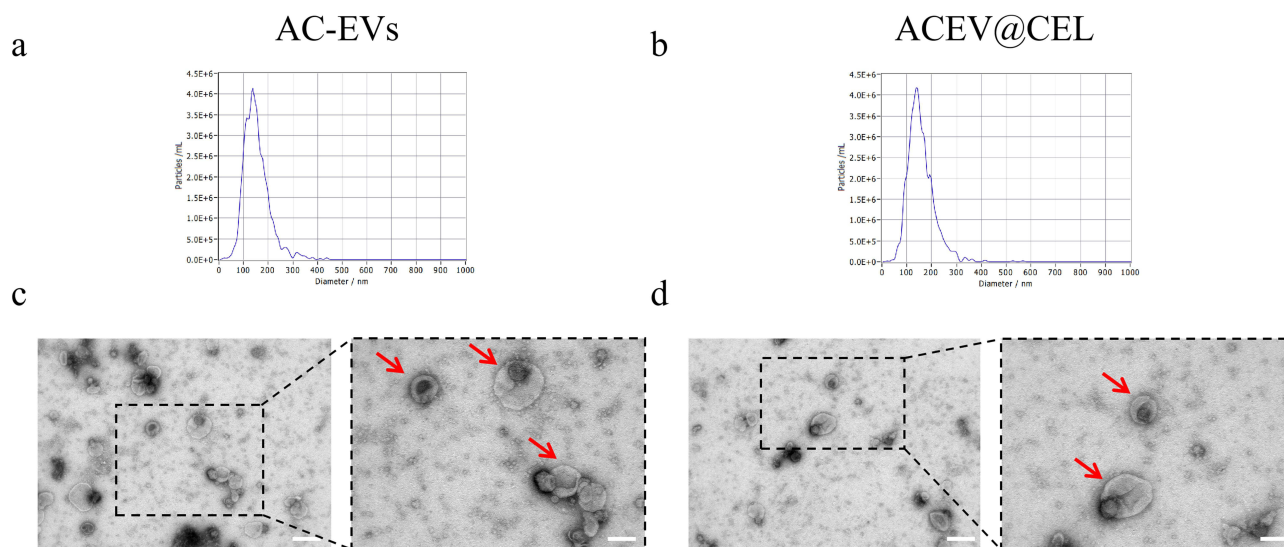


Figure 2 Characterization of AC-EVs and ACEV@CEL by NTA and TEM. (a and b) NTA showing the particle size distribution of AC-EVs and ACEV@CEL, respectively. (c and d) Transmission electron microscopy (TEM) images illustrating the morphology and size of AC-EVs and ACEV@CEL. Scale bars: 200 nm (left panels) and 100 nm (right panels). Red arrows indicate EVs.

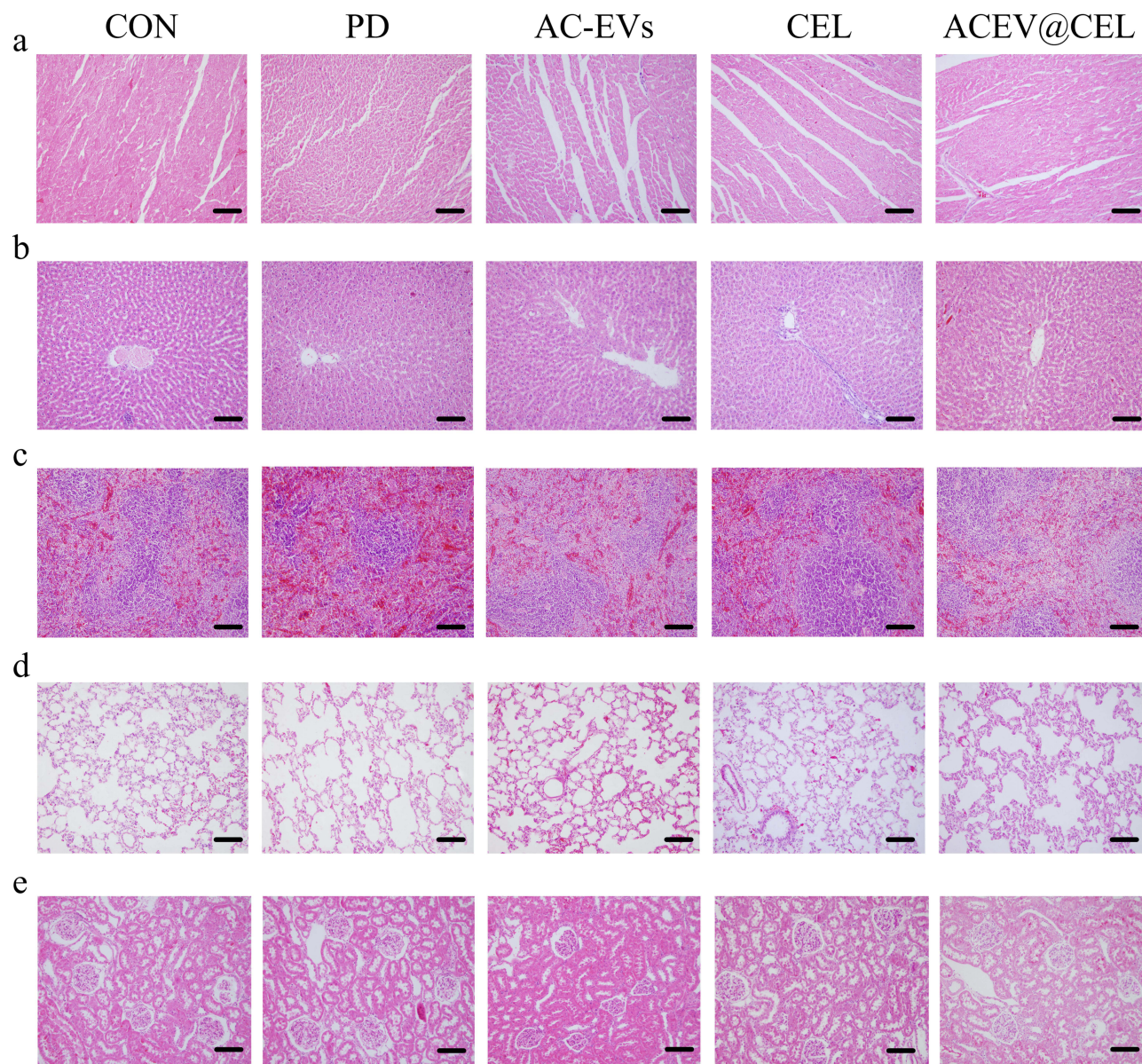


Figure 3 In vivo biocompatibility evaluation of different treatments. Representative H&E staining images of major organs: (a) heart, (b) liver, (c) spleen, (d) lung, and (e) kidney from different groups (CON, PD, AC-EVs, CEL, and ACEV@CEL). No significant histopathological abnormalities were observed. Scale bar = 100 μ m.

and 96–219 nm (Figure 2b), respectively. The particle concentration of the AC-EVs group was approximately 8.3×10^7 particles/mL, while that of the ACEV@CEL group was approximately 8.4×10^7 particles/mL. Furthermore, the morphology of the EVs was further characterized by TEM. As observed, both AC-EVs and ACEV@CEL exhibited a typical vesicular structure with a well-defined lipid bilayer membrane. The vesicles predominantly presented a round to oval morphology, with some displaying a characteristic cup-shaped appearance, which is commonly attributed to dehydration during sample preparation. The membrane boundary was clearly visible and intact, indicating good structural integrity after isolation and drug loading. No obvious aggregation, fragmentation, or structural collapse was observed in either group, suggesting that the loading process did not adversely affect vesicle morphology.

Biocompatibility of AC-EVs and ACEV@CEL in Rats

Biocompatibility is critical for the local application of therapeutic agents. Therefore, histopathological evaluations of major organs, including the heart, liver, spleen, lungs, and kidneys, were performed to assess inflammatory infiltration

and tissue damage across all groups (Figures 3a–e). H&E staining showed that myocardial fibers in all groups were well-organized and arranged in bundles, with no evident edema or inflammatory cell infiltration (Figure 3a). In the liver, hepatocytes displayed normal morphology with clear nuclei, radially arranged hepatic cords, and no signs of inflammatory infiltration, steatosis, or necrosis (Figure 3b). The spleen exhibited clear boundaries between red and white pulp, with intact lymphoid structures and no evidence of hemorrhage or necrosis (Figure 3c). In the lungs, alveolar structures were intact without collapse or overexpansion, and no edema, congestion, or inflammatory infiltration was observed (Figure 3d). Similarly, renal tissues showed intact glomerular structures without atrophy or swelling, well-organized renal tubules with preserved epithelial integrity, and no signs of hemorrhage or necrosis in the interstitium (Figure 3e). Collectively, these findings indicate that none of the treatments induced detectable damage to major organs, confirming the favorable *in vivo* biocompatibility of the administered formulations at the tested doses and frequencies.

ACEV@CEL Inhibits Alveolar Bone Resorption in Periodontitis Rats

To evaluate alveolar bone loss among different groups, Micro-CT was performed on rat maxillae. Representative three-dimensional, H&E staining and TRAP staining figures are shown in Figures 4a–d. In the CON group, the alveolar bone surrounding the maxillary first molar and the region between the first and second molars remained largely intact, with no evident bone loss or structural disruption. In contrast, the PD group exhibited pronounced crater-like bone resorption, particularly at the ligature site, accompanied by irregular bone surfaces. Compared with the PD group, alveolar bone loss was alleviated in the AC-EVs, CEL, and ACEV@CEL groups, with the ACEV@CEL group showing the most pronounced protective effect. Consistently, H&E staining of alveolar bone tissues was performed to identify the positions of the ABC and the CEJ between the first and second molars. The CON group exhibited the shortest CEJ–ABC distance, indicating minimal bone loss, whereas this distance was markedly increased in the PD group, reflecting severe bone resorption. All treatment groups showed varying degrees of recovery, with the ACEV@CEL group demonstrating the greatest improvement. Quantitative analysis further confirmed that the CEJ–ABC distance was significantly increased in the PD group compared with the CON group ($p < 0.001$), indicating successful establishment of the periodontitis model. Treatment with AC-EVs, CEL, and ACEV@CEL significantly reduced this distance compared with the PD group ($p < 0.01$, $p < 0.01$, and $p < 0.001$, respectively). Notably, the ACEV@CEL group exhibited a significantly shorter CEJ–ABC distance than both the AC-EVs and CEL groups ($p < 0.05$), suggesting superior inhibition of alveolar bone resorption (Figure 4e).

Furthermore, TRAP staining revealed that osteoclasts were identified as multinucleated (≥ 3 nuclei) pink-stained cells. Quantitative analysis of osteoclast numbers in regions surrounding the first molar roots and between the first and second molars showed that the PD group had the highest osteoclast count, indicating enhanced osteoclastogenesis and bone resorption. In contrast, all treatment groups exhibited significantly reduced osteoclast numbers, with the ACEV@CEL group showing fewer osteoclasts than both the AC-EVs and CEL groups ($p < 0.05$). (Figure 4f) These results demonstrate that ACEV@CEL effectively suppresses osteoclast differentiation and exhibits superior efficacy compared with AC-EVs or celecoxib alone. Collectively, ACEV@CEL significantly inhibits alveolar bone resorption and osteoclastogenesis in periodontitis rats, outperforming the individual components.

Internalization of AC-EVs and ACEV@CEL

In vitro, the uptake of AC-EVs and ACEV@CEL by RAW264.7 cells was assessed using confocal laser scanning microscopy. As shown in Figure 5, cell nuclei were stained blue, the cytoskeleton was stained green, and Dil-labeled AC-EVs and ACEV@CEL exhibited red fluorescence. These vesicles appeared as punctate signals localized within the cytoplasm of RAW264.7 cells, indicating successful cellular internalization. This finding provides a structural basis for their subsequent intracellular biological functions.

CCK-8 Assay for Cell Viability

To determine the optimal concentrations for *in vitro* experiments, RAW264.7 cells were co-cultured with different concentrations of AC-EVs, celecoxib, and ACEV@CEL. In the AC-EVs group, cell viability remained close to 100% across concentrations ranging from 5 to 100 $\mu\text{g/mL}$, indicating negligible cytotoxicity. In the celecoxib group, cell

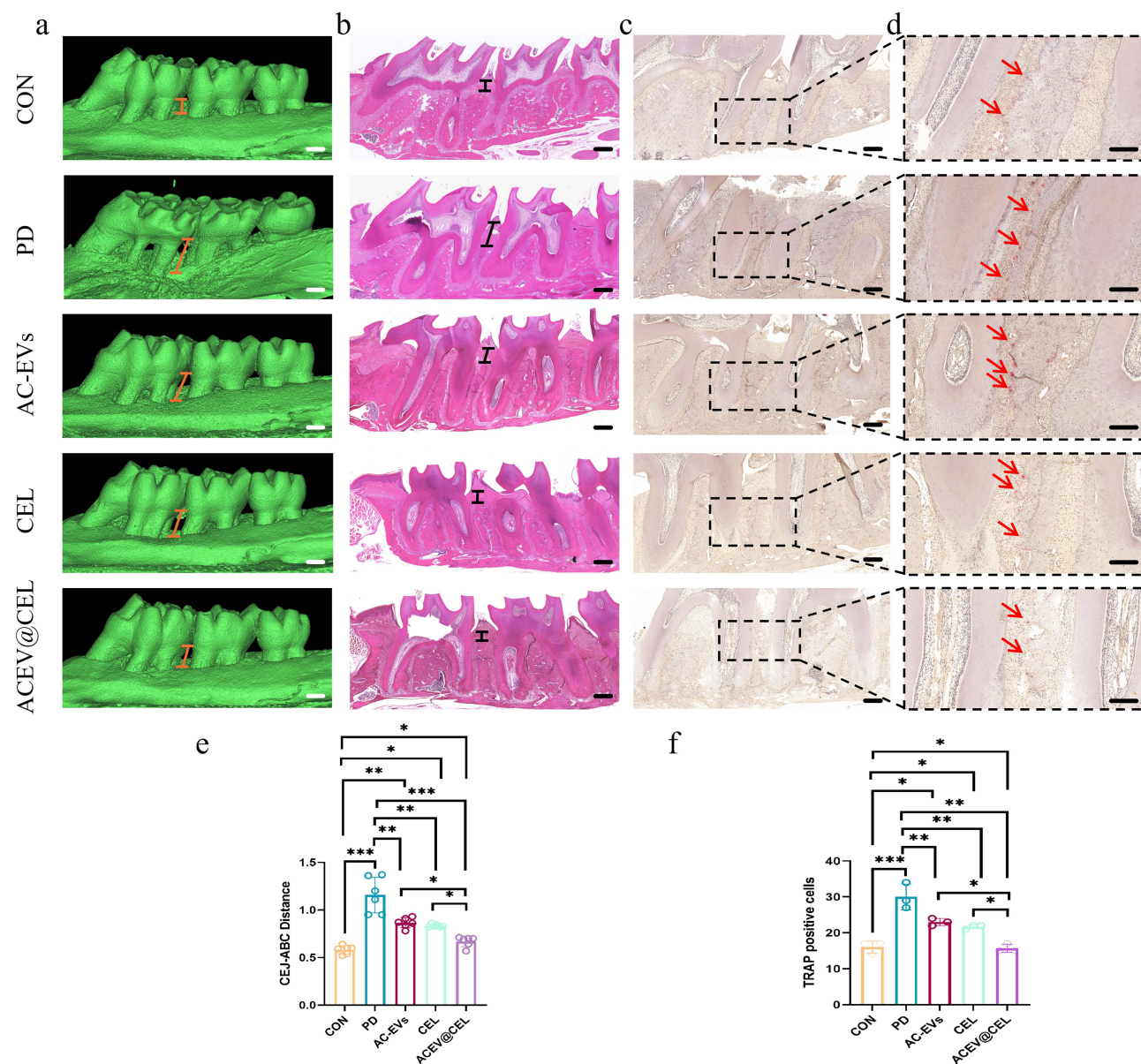


Figure 4 In vivo assessment of alveolar bone resorption in periodontitis rats. (a) Micro-CT images and three-dimensional reconstruction of rat maxillary alveolar bone. The labeled region indicates the distance from the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) between the first and second maxillary molars. Scale bar = 200 μ m. (b) Representative H&E staining of rat maxilla. Scale bar = 200 μ m. (c and d) TRAP staining observed at 100 $\times$ (c) and 400 $\times$ (d) magnification. Multinucleated, TRAP-positive osteoclasts (≥ 3 nuclei) are stained pink, as indicated by arrows. Scale bar = 200 μ m. (e and f) Quantitative analysis of the CEJ-ABC distance (e) and the number of TRAP-positive cells (f). Data are presented as mean $\pm$ SD. $n = 6$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

viability was approximately 100% at 1, 5, and 10 μ M, while slight decreases were observed at 20 and 50 μ M; however, viability remained above 80%, suggesting no significant cytotoxic effects. Similarly, when the celecoxib content within ACEV@CEL was adjusted to equivalent concentrations (1–50 μ M), cell viability remained around 100% (Figure 6), confirming the excellent in vitro biosafety of ACEV@CEL at these concentrations.

TRAP Staining for Osteoclast Differentiation

To evaluate the effects of AC-EVs, celecoxib, and ACEV@CEL on osteoclast differentiation, TRAP staining was performed. Osteoclasts were identified as large, multinucleated (≥ 3 nuclei) cells with a characteristic vacuolated morphology and positive pink staining (Figure 7a). Quantitative analysis (Figure 7b) showed that the CON group exhibited the highest number of osteoclasts, whereas all treatment groups showed reduced osteoclast formation,

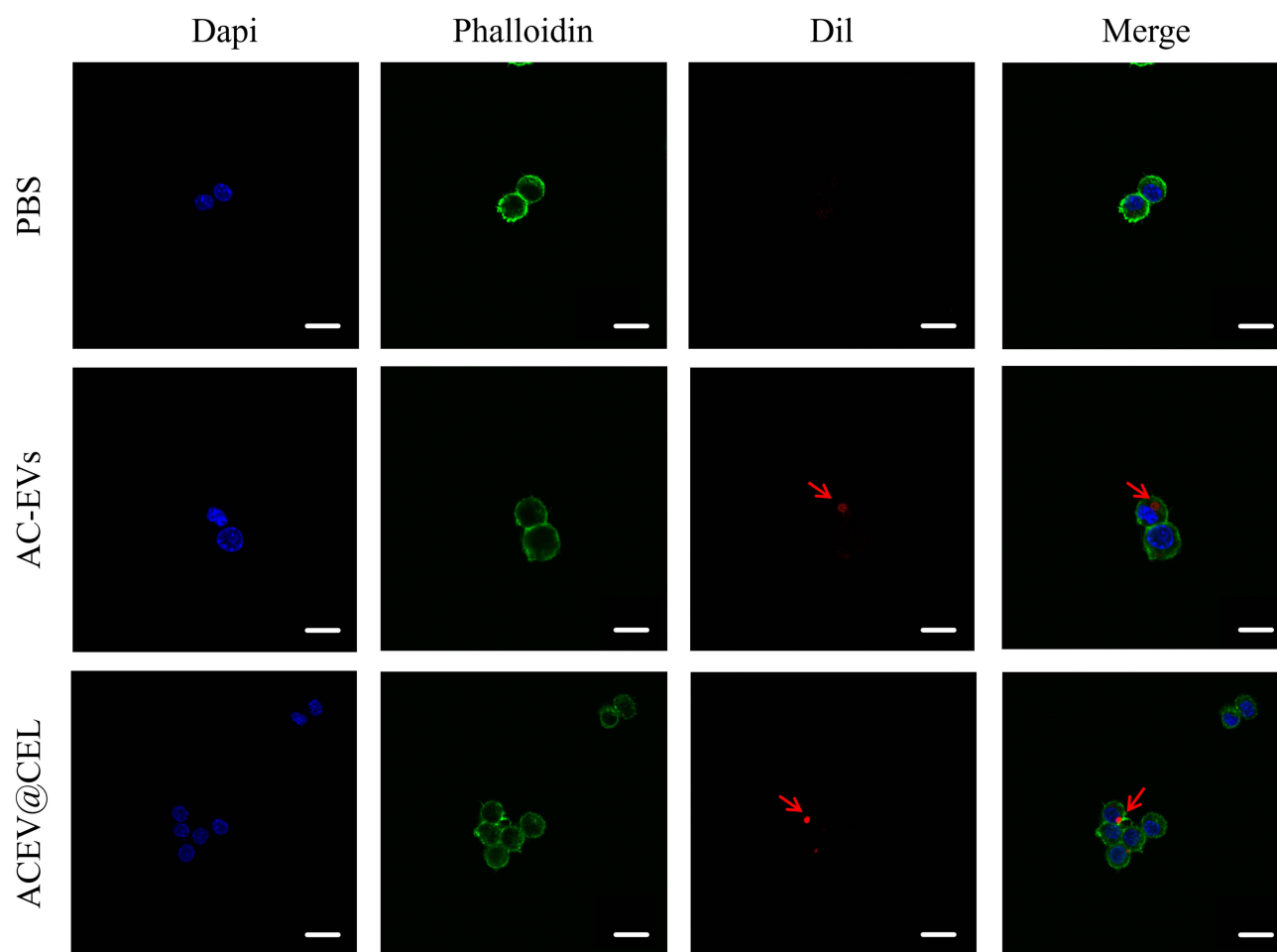


Figure 5 In vitro cellular uptake of AC-EVs and ACEV@CEL by RAW264.7 cells. After stimulation, RAW264.7 cells were co-incubated with Dil-labeled AC-EVs or ACEV@CEL for 4 h. Cellular internalization was observed using laser scanning confocal microscopy. AC-EVs and ACEV@CEL are shown in red. Scale bar = 20 μ m.

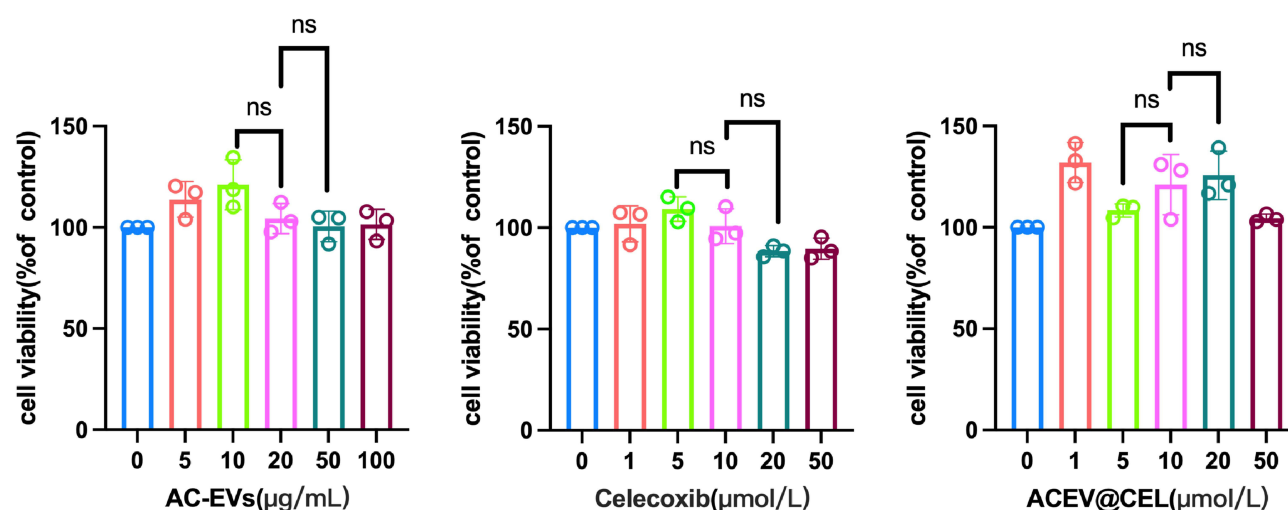


Figure 6 In vitro cytotoxicity of AC-EVs, celecoxib, and ACEV@CEL. Cell viability of RAW264.7 cells treated with different concentrations of AC-EVs, celecoxib, and ACEV@CEL was assessed using the CCK-8 assay. Data are presented as mean $\pm$ SD. ns: no significant difference.

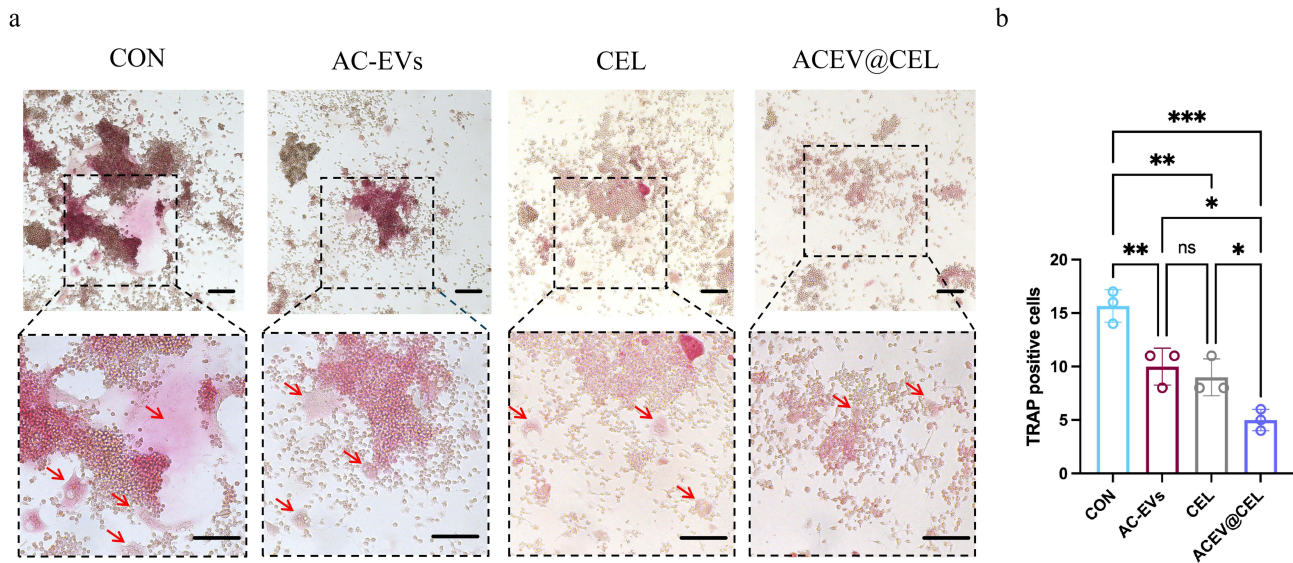


Figure 7 TRAP staining for evaluation of osteoclast differentiation in vitro. (a) Representative images of osteoclasts at 100× (upper) and 200× (lower) magnification. Osteoclasts containing ≥3 nuclei are stained pink (indicated by red arrows). Scale bar = 100 μm. (b) Quantitative analysis of osteoclast numbers in each group. Data are presented as mean ± SD (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001; ns: no significant difference.

indicating inhibitory effects on osteoclast differentiation. No significant difference was observed between the AC-EVs and CEL groups. However, the ACEV@CEL group exhibited a significantly lower number of osteoclasts compared with both groups ($p < 0.05$), suggesting a stronger inhibitory effect.

Western Blot Analysis of Osteoclast-Related Proteins

During osteoclast differentiation, TRAF6 mediates signal transduction and initiation, NFATc1 serves as a master transcription factor driving osteoclast-specific gene expression, and CTSK acts as a terminal effector protein and a hallmark of mature osteoclasts. Together, these markers represent different stages of osteoclastogenesis. Western blot analysis (Figure 8a) demonstrated that, compared with the CON group, the expression levels of CTSK, TRAF6, and NFATc1 were significantly downregulated in both the CEL and ACEV@CEL groups, indicating inhibition of osteoclast differentiation. In the AC-EVs group, CTSK expression showed a decreasing trend without statistical significance, whereas TRAF6 and NFATc1 were significantly reduced compared with the CON group ($p < 0.05$). Notably, compared with the AC-EVs and CEL groups, ACEV@CEL treatment resulted in a more pronounced downregulation of all three proteins, further confirming its superior inhibitory effect on osteoclast differentiation. (Figure 8b)

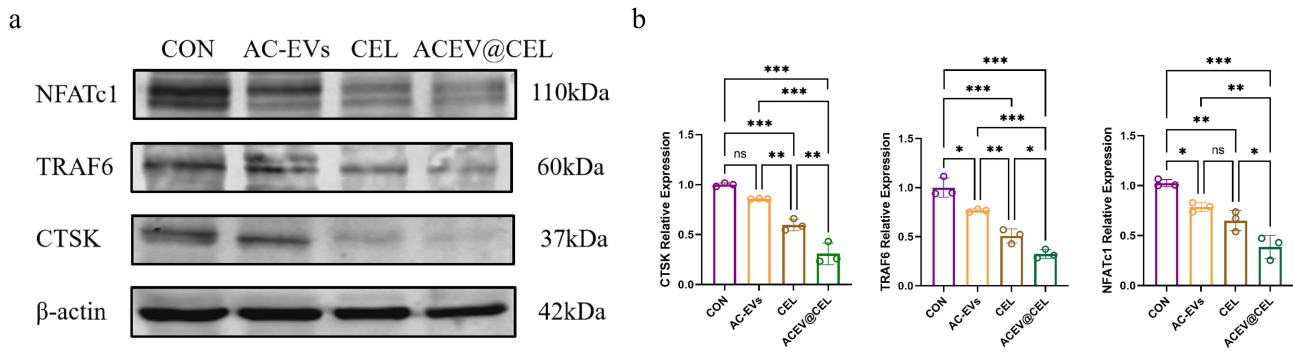


Figure 8 Western blot analysis of CTSK, TRAF6, and NFATc1 expression in osteoclasts. (a) Representative bands and corresponding quantitative analysis. Protein expression levels were normalized to β-actin. (b) Quantitative results are presented as mean ± SD (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001; ns: no significant difference.

Discussion

In this study, we isolated and purified AC-EVs and subsequently loaded the small-molecule drug celecoxib into AC-EVs via ultrasonication combined with co-incubation to generate ACEV@CEL. In vivo experiments demonstrated that local administration of ACEV@CEL into the gingival sulcus effectively inhibited alveolar bone resorption in rats with periodontitis, while exhibiting favorable biocompatibility. Meanwhile, in vitro studies showed that ACEV@CEL could be internalized by cells, exert biological functions, and suppress the differentiation of RAW264.7 cells into osteoclasts.

Unlike MDEVs, PDEVs originate from more complex biological sources. Plant cells contain cell walls and chloroplasts, which, upon disruption, release various polysaccharides and proteins that may compromise the purity of the isolated vesicles. Therefore, we employed a combination of differential centrifugation, sucrose density gradient centrifugation, and ultracentrifugation to purify AC-EVs, effectively reducing plant-derived contaminants.³⁷ As shown in Figure 2, morphological analysis by TEM confirmed that both AC-EVs and ACEV@CEL exhibited a well-defined lipid bilayer structure with a typical spherical morphology. NTA revealed that the particle sizes of both vesicles were distributed within the characteristic size range of EVs,³⁸ with only minor differences between the two groups. This indicates that the loading process did not induce significant drug aggregation or vesicle disruption, suggesting good controllability of the preparation method. In addition, the stability in morphology and particle size enables both vesicle types to maintain structural integrity under different conditions, highlighting their potential advantages for in vivo applications. It is worth noting that, in contrast to MDEVs, the protein composition of PDEVs is highly influenced by plant species and isolation methods, and there is currently no universally recognized specific surface protein marker. Although certain plant-specific proteins have been proposed, their presence still requires validation across multiple plant sources.³⁹

The biosafety of local delivery systems is a critical factor limiting their clinical translation,⁴⁰ particularly for nanocarriers loaded with small-molecule drugs, where nonspecific drug distribution and potential immunogenicity of the carrier may lead to systemic toxicity.⁴¹ In the rat periodontitis model, we administered AC-EVs, celecoxib, and ACEV@CEL via local injection into the gingival sulcus to evaluate their effects on alveolar bone resorption. We first assessed the impact of these treatments on major organs. As shown in Figure 3, H&E staining revealed no observable histopathological damage in distant vital organs, indicating that ACEV@CEL can maintain structural integrity in vivo and undergo relatively mild degradation. Moreover, encapsulation of hydrophobic drugs within vesicular structures may reduce the exposure of free drugs in systemic circulation, thereby lowering the risk of damage to metabolic organs. This phenomenon has also been reported in other studies involving drug-loaded PDEVs.^{42,43} Therefore, AC-EVs show great potential as drug delivery carriers.

To further evaluate the therapeutic efficacy of ACEV@CEL in inhibiting alveolar bone resorption in periodontitis, we performed a comprehensive assessment using multiple approaches, including Micro-CT three-dimensional reconstruction, H&E staining, and TRAP staining (Figure 4). Compared with the untreated periodontitis model, all treatment groups exhibited varying degrees of inhibition of bone resorption. From an overall perspective of bone structural changes, the improvement in alveolar bone loss following local gingival administration is unlikely to be driven by a single factor, but rather results from the synergistic effects of inflammation suppression and regulation of bone metabolism. Free celecoxib partially alleviated bone resorption, suggesting that it may inhibit COX-2-mediated production of prostaglandin E2 (PGE2), which is closely associated with osteoclastogenesis.^{44,45} However, its therapeutic efficacy remained limited, possibly due to rapid local diffusion, a short half-life, and insufficient maintenance of effective local concentrations. Similarly, the AC-EVs group also demonstrated a certain degree of bone-protective effect, indicating that AC-EVs themselves possess the potential to modulate bone metabolism under inflammatory conditions, likely attributable to their intrinsic bioactive components derived from onion. Notably, compared with the AC-EVs and CEL groups, the ACEV@CEL group exhibited more pronounced improvements in alveolar bone height and reductions in osteoclast numbers, suggesting a synergistic enhancement in inhibiting bone resorption. This enhanced effect may arise from two complementary mechanisms: on one hand, EVs, as natural nanocarriers, can improve the retention and delivery efficiency of celecoxib at inflammatory sites, thereby maintaining stable drug concentrations, consistent with the biological properties observed in other EV-based delivery systems;^{46,47} on the other hand, the intrinsic inhibitory effect

of AC-EVs on bone resorption, as evidenced in the AC-EVs group, further contributes to the superior therapeutic outcome of the combined system.

Although the *in vivo* findings demonstrated that ACEV@CEL effectively attenuated alveolar bone resorption in periodontitis, the underlying regulatory mechanisms on osteoclast differentiation required further validation. Therefore, we conducted complementary *in vitro* experiments. Confocal microscopy tracking revealed that both AC-EVs and ACEV@CEL were efficiently internalized by RAW264.7 cells (Figure 5). This observation provides a prerequisite for their biological activity at the cellular level and further supports that EVs, as natural nanostructures, not only maintain structural stability through their lipid bilayer but also facilitate intracellular delivery via interactions with the cell membrane.^{48,49} This property enables efficient transport of therapeutic agents into target cells, thereby laying the foundation for subsequent regulation of osteoclast differentiation.

Considering that drug-induced effects on cellular function may be confounded by cytotoxicity, we first evaluated the biocompatibility of the three treatments prior to assessing their effects on osteoclast differentiation. To ensure rational experimental design, we referred to commonly reported concentration ranges in the literature (approximately 20 µg/mL for EVs and 10 µM for celecoxib)^{50–53} and established gradient concentrations accordingly (Figure 6). CCK-8 assay results showed that AC-EVs exerted negligible effects on cell viability even at concentrations up to 100 µg/mL, demonstrating excellent *in vitro* biosafety. For celecoxib, cell viability remained unaffected at concentrations below 10 µM, whereas a reduction in viability was observed at 20 µM and 50 µM. In contrast, when celecoxib was loaded into AC-EVs at equivalent concentrations (20 µM and 50 µM), no significant cytotoxicity was detected. These findings suggest that the incorporation of AC-EVs may mitigate the transient high local exposure of celecoxib within cells, thereby buffering adverse cellular responses and improving the overall biocompatibility of ACEV@CEL.

Upon stimulation of monocyte–macrophage lineage cells, the binding of RANKL to its receptor RANK leads to the initial assembly of the membrane-proximal TRAF6 signaling complex, which transduces extracellular signals into downstream pathways, including NF-κB, MAPK/AP-1, and PLCγ-Ca²⁺ signaling cascades. Subsequently, NFATc1 is induced, undergoes nuclear translocation, and amplifies its own expression, thereby initiating the “differentiation–fusion–maturation” process of osteoclast precursors.⁵⁴ In the later stages, CTSK, as a terminal effector gene regulated by NFATc1, is highly expressed and specifically transported to the bone resorption lacuna, where it mediates degradation of the organic bone matrix.⁵⁵ In the present study, at the functional level, all treatment groups reduced the formation of TRAP-positive cells, with the ACEV@CEL group exhibiting the most pronounced inhibitory effect. This enhanced inhibition may be attributed to the improved drug delivery efficiency and sustained local drug concentration provided by EVs, thereby amplifying their regulatory effects on osteoclast differentiation. At the molecular level, although a decreasing trend in CTSK expression was observed in the AC-EVs group, the difference was not statistically significant. Combined with TRAP staining results, this may be related to insufficient stimulation duration, which may not have reduced CTSK expression to a detectable extent. Consistent with the *in vivo* findings, the ACEV@CEL group exhibited the lowest osteoclast numbers and the most significant downregulation of osteoclast-related proteins, indicating a synergistic mechanism between AC-EVs and celecoxib in suppressing osteoclast differentiation. Collectively, this combined delivery system enhances the inhibition of osteoclastogenesis through multi-level regulatory effects.

Limitations and Future Perspectives

Nevertheless, this study has several limitations. First, regarding EVs characterization, although TEM and NTA were employed to verify morphology and size distribution, additional approaches such as Western blotting, spectrophotometric analysis, or proteomics have not yet been applied to systematically identify EVs surface proteins. Given that PDEVs currently lack universally recognized specific markers, such complementary analyses would further improve the accuracy of vesicle identification. Moreover, the functional evaluation of EVs in this study primarily focused on particle size and morphological characteristics, without in-depth investigation of their ultrastructural features or subpopulation heterogeneity such as zeta potential analysis, which may limit a comprehensive understanding of the relationship between structure and function. Second, although this study demonstrates that AC-EVs can serve as an effective delivery vehicle for celecoxib and exert inhibitory effects on osteoclast differentiation, the precise molecular mechanisms underlying drug delivery remain to be fully elucidated. In particular, the interactions between EVs and recipient cells, as well as the

regulation of downstream signaling pathways, require further investigation. In addition, further validation through higher-level animal studies, well-designed clinical investigations and a long-term observation of efficacy will be necessary to confirm the translational potential of this therapeutic strategy.

From an application perspective, future studies should focus on optimizing drug-loading efficiency and improving delivery stability. This strategy, which combines naturally derived EVs with conventional therapeutics, holds promise for extension to the treatment of other bone-related diseases, thereby broadening its clinical applicability and providing both a theoretical foundation and practical basis for the development of next-generation nanodrug delivery systems.

Conclusions

In this study, AC-EVs were extracted, and celecoxib was loaded into them through sonication and co-incubation to obtain ACEV@CEL. In vivo and in vitro experiments demonstrated that ACEV@CEL could inhibit osteoclast differentiation in the periodontitis environment. Its effect was stronger than that of ACEVs or celecoxib alone. Moreover, ACEV@CEL exhibited good biocompatibility, making it a promising candidate for further research as a potential drug for treating alveolar bone resorption in periodontitis.

Data Sharing Statement

Data available on request from authors.

Ethics Approval and Informed Consent

All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and the ARRIVE 2.0 guidelines, and were approved by the Animal Ethics Committee of China Medical University (Approval No. CMU20241371).

Consent for Publication

All authors agree to submit for publication in “International Journal of Nanomedicine”.

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Disclosure

The authors declare no competing interests in this work.

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