

Gelatin macromolecular microspheres constructed by microfluidics regulate the TNF/HIF-1 signaling axis to reshape the bone immune microenvironment and enhance infectious bone defect repair

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ABSTRACT

Background: Infected bone defects are difficult to treat due to bacterial biofilms and chronic inflammation. Systemic antibiotics often fail and cause severe side effects. A dual-drug delivery system that combines anti-bacterial and osteo-immunomodulatory actions may offer a synergistic solution.

Methods: A gelatin-based microfluidic microsphere (GM@RES@VAN) co-loading vancomycin (VAN) and resveratrol (RES) was developed. The microspheres were characterized for morphology, drug release, and elemental composition. Antibacterial and biofilm-degrading effects were assessed in vitro against MRSA. A rat infected bone defect model was used to evaluate in vivo healing via micro-CT, histology, and immunofluorescence. Network pharmacology, molecular docking, and cellular assays (qPCR, Western blot, TEM, HIF inhibition) explored the molecular mechanism.

Results: GM@RES@VAN microspheres showed uniform size, smooth surface, and sustained dual-drug release. In vitro, the microspheres exhibited potent bactericidal activity and biofilm degradation (**p < 0.001). In vivo, the GM@RES@VAN group significantly reduced local bacterial load, decreased inflammatory infiltration, and promoted collagen deposition and new bone formation, with increased bone mineral density and bone volume/tissue volume (*p < 0.05). Mechanistically, resveratrol suppressed pro-inflammatory factors (IL6, TNF, IL1B), reduced HIF1A, MMP9, and PTGS2 expression, preserved mitochondrial ultrastructure, upregulated osteogenic markers (ALP, OCN, COL I) and the anti-apoptotic protein BCL2. Vancomycin primarily cleared the infection, while resveratrol acted through the TNF/HIF-1 axis to reshape the inflammatory microenvironment and promote osteogenesis—their synergy was confirmed by HIF inhibition tests.

Conclusion: The GM@RES@VAN dual-drug microspheres achieve programmed antibacterial and osteo-immunomodulatory effects via vancomycin-mediated infection clearance and resveratrol-driven TNF/HIF-1 pathway regulation. This combination offers a promising strategy for treating refractory infected bone defects.

1. Introduction

In orthopaedics, infectious bone defects are among the most

damaging conditions. They are usually caused by high-energy trauma, diabetic foot, postoperative infection after internal fixation, and other factors. The intricate interplay of bone tissue continuity disruption,

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pathogen colonization, and biofilm development constitutes its clinical hallmark [1]. Globally, with the aging of the population, the popularization of the application of implants and the emergence of drug-resistant strains, its incidence rate and treatment difficulty are continuously rising [2]. Comprehensive debridement, long-term systemic antibiotic use, and subsequent bone transplantation or bone transport surgery are the mainstays of the conventional treatment approach for such defects. However, these approaches have numerous drawbacks, including lengthy treatment cycles, high systemic toxicity, frequent infection recurrence, and poor regeneration efficiency [3,4]. Among these, systemic antibiotic administration makes it challenging to reach effective concentrations in ischemia sclerosis bone infection foci and may instead result in bacterial resistance; However, harsh inflammatory and infectious microenvironments frequently significantly reduce the osteogenic efficiency of a single bone graft material [5–7]. As a result, creating a dual functional delivery system of “antibacterial osteogenic” that can accomplish cooperative and sustained administration in the defect area has emerged as a critical therapeutic need and research hotspot in this sector. A new approach to the problem of infectious bone defects is provided by localized drug delivery systems, particularly microspheres based on advanced manufacturing technology, which can precisely deliver drugs to lesions and achieve controlled release. This is anticipated to significantly increase local drug concentration while reducing systemic side effects [8–10].

The selection of drugs and the design of carriers are essential components of a successful local delivery system. Regarding medications, we have creatively embraced a cooperative approach that blends Western and traditional Chinese medicine. As a glycopeptide antibiotic, vancomycin is the “last line of defense” against serious infections brought on by Gram positive bacteria, particularly MRSA (methicillin-resistant *Staphylococcus aureus*). Bone regeneration barriers can be removed by its potent bactericidal properties [11,12]. However, starting and finishing high-quality bone restoration requires more than just antimicrobial treatments. Resveratrol, a naturally occurring monomeric molecule that comes from plants used in traditional Chinese medicine, like grapes and *Polygonum cuspidatum*, has garnered a lot of interest lately because of its superior biological capabilities [13,14]. The research team's earlier studies have demonstrated that resveratrol not only possesses anti-inflammatory and antioxidant stress resistance properties, but it also directly stimulates osteoblast differentiation and bone matrix mineralization through a variety of signaling pathways and has a specific promoting effect on angiogenesis [13,15,16]. It is theoretically possible to create a synergistic therapeutic alliance that can both eradicate infections and encourage regeneration by combining the potent antibacterial qualities of vancomycin with the potent anti-inflammatory/bone-promoting capacity of resveratrol. In order to create structured gelatin-based drug-loaded microspheres, we opted for microfluidic technology [17,18].

Based on the above background, this study aims to develop and systematically evaluate a dual drug microsphere of resveratrol/vancomycin based on microfluidic technology (GM@RES@VAN). And further explore its role and molecular mechanism in repairing infectious bone defects. The implementation of this study not only provides a new type of integrated traditional Chinese and Western medicine treatment product with clinical translation potential for infectious bone defects, but also profoundly reveals the new mechanism of local drug delivery system regulating the bone immune microenvironment to promote functional regeneration from the multiple interfaces of “material cell molecule”. It has important theoretical significance and application value for promoting the development of bone repair materials towards “intelligence” and “functionalization”.

2. Materials and methods

2.1. GM@RES@VAN synthesis and structural characterization of composite drug loaded microspheres

2.1.1. GM@RES@VAN synthesis of drug loaded microspheres

Synthesis of gelatin microspheres loaded with both resveratrol and vancomycin using microfluidic technology (GM@RES@VAN: GelMA was dissolved in PBS at 37 °C to prepare 5–10% (w/v) aqueous phase, and the photoinitiator LAP was added at 0.05–0.10% (w/v) [19,20]. Resveratrol (RES) is first dissolved in a small amount of ethanol and then added (the volume fraction of ethanol in the aqueous phase is controlled to be $\leq 10\%$), with a final concentration of 1 mg/mL. The final concentration of vancomycin (VAN) is 5 mg/mL. Add Span-80 1.0–1.5% (w/v) as a surfactant to liquid paraffin (mineral oil) for oil phase selection. After assembling the coaxial glass capillary microfluidic chip (inner tube inner diameter of 200 μm , outer tube inner diameter of 500 μm), samples were injected at flow rates of 5 $\mu\text{L}/\text{min}$ (dispersed phase) and 20 $\mu\text{L}/\text{min}$ (continuous phase), respectively, to generate uniform water in oil (W/O) droplets at the chip outlet. Droplets are subjected to 405 nm LED light irradiation for 30–60 s at the outlet or in a collection bottle (in an ice bath, away from light) to complete GelMA photo crosslinking curing. Add 2–3 vol of isopropanol (IPA, cold) to the collected lotion, gently invert and mix for 30–60 s, and then stand at room temperature/centrifuge (1000–1500 g, 3–5 min) to remove the supernatant oil phase; Repeat 1–2 times until the supernatant is essentially clear and there is no obvious oil phase. Wash the microspheres sequentially with 100% IPA (rapid, $\leq 30\text{s}$), 70% IPA (once), deionized water (once), and PBS (three times) until there is no oil odor and no signs of foaming on the surface. Freeze dry the washed microspheres ($-50\text{ }^{\circ}\text{C}$, 10 Pa, 24 h) to obtain a dry powder, and store it in the dark at $-20\text{ }^{\circ}\text{C}$; Rehydrate with PBS before use. Place 2 mg of drug loaded microspheres into a dialysis bag (with a cut-off molecular weight of 3500 kDa), immerse them in PBS (pH 7.4) containing 2 $\mu\text{g}/\text{mL}$ hyaluronidase, and shake at 37 °C. Sample 2 mL at the set time point and add an equal amount of fresh PBS. After treating the sample with ethanol and centrifuging, measure the concentrations of RES and VAN in the supernatant, and plot the in vitro release curve. Take a quantitative amount of pre degradation microspheres, dry and weigh them (W_0), and degrade them under specific conditions. After reaching the scheduled time, take out the sample and dry it thoroughly, then weigh it again (W_t). The weight ratio of its degradation participation (%) = $(W_0 - W_t)/W_0 \times 100\%$. This ratio quantifies the quality loss of the material during the degradation process. GelMA and LAP solutions are filtered through a 0.22 μm membrane for sterilization; Resveratrol and vancomycin mother liquor were also filtered using a 0.22 μm membrane. The oil phase (liquid paraffin + Span-80) was sterilized by high-pressure steam (121 °C, 20 min). All operations are carried out in a biosafety cabinet. The isopropanol, PBS, and other sterile reagents used in the preparation process; The washing and collection steps are carried out in a sterile environment. After freeze-drying, the final microspheres were stored in the dark at $-20\text{ }^{\circ}\text{C}$ and rehydrated with sterile PBS before use.

2.1.2. Characterization of microsphere structure

50 microspheres were chosen at random, their diameters were recorded using ImageJ, and their distribution was statistically examined using a bright field microscope to view the general morphology and dispersion of microspheres. To confirm whether the medicine was loaded, the surface and interior structure of microspheres were examined at 200 μm and 50 μm scales using scanning electron microscopy. Utilizing an energy spectrometer, do elemental analysis to ascertain composition and distribution.

3. GM@RES@VAN - determination of antibacterial properties of drug loaded microspheres

Grouping settings: Control group (MRSA bacterial coated plate only), GM group (MRSA bacterial coated plate and blank gelatin microspheres) GM@RES (MRSA bacterial coated plates and resveratrol gelatin microspheres) GM@RES@VAN (MRSA bacterial coated plates and dual drug microspheres), repeated 3 times per group.

3.1. Bacterial coating experiment

This study cultured samples from each treatment group with MRSA bacterial solution at a concentration of 1×10^6 CFU/mL for 24 h under appropriate circumstances in order to assess the antibacterial activity of various treatment groups against MRSA. Following incubation, take 100 μ L of each group's bacterial solution and use PBS to make a series of gradient dilutions. The bacterial solution with a particular dilution was then uniformly applied to the agar plate's surface and incubated for 24 h at 37 °C in an incubator with a constant temperature. Once the cultivation is finished, precisely count the colony forming units (CFU) on the plate, determine the number of colonies in each group, and assess the antibacterial action quantitatively.

3.2. Bacterial viability staining experiment

Following co-incubation, we employed the Calcein AM and Propidium Iodide (PI) double staining method to detect and statistically analyze bacterial activity in order to precisely assess each experimental group's antibacterial impact against MRSA. Add the proper amount of calcein AM/PI staining solution to each reaction system following a 24-h co-incubation with bacterial solution. To guarantee adequate interaction between the dye and bacteria, properly mix the sample after adding the solution, then incubate it in the dark at 37 °C for 30 min. Immediately after incubation, transfer 10 μ L of the bacterial solution onto a glass slide, seal it with a slide seal, and examine it under a fluorescence microscope. Observe and gather pictures under the green and red fluorescence channels independently using the proper filter groups. Lastly, the green fluorescence (living bacteria) and red fluorescence (dead bacteria) signals in the field of view were counted using image analysis software. The ratio of live to dead bacteria was then calculated, and the antibacterial efficacy of each treatment group was quantitatively assessed.

3.3. Biofilm degradation experiment

We created the following experimental paradigm in order to assess each experimental group's effects on mature MRSA biofilm breakdown and clearance: To create a durable, mature biofilm, a standardized MRSA bacterial solution was first added to a 96-well cell culture plate and cultured at 37 °C for 48 h. The planktonic bacteria and culture media should then be thoroughly aspirated from each well. To get rid of non-adherent bacteria, gently wash the biofilm surface twice with sterile PBS buffer. Following the aforementioned preprocessing, each group of wells received fresh culture media with the appropriate intervention medications, and the intervention culture was maintained at 37 °C for a full day. After the intervention, discard the liquid in the well and wash gently. Then add 1% crystal violet staining solution and stain in the dark at room temperature for 15-30 min. After dyeing, thoroughly wash away the unbound dye with deionized water. After the well plate is naturally dried, add 95% ethanol solution to dissolve and decolorize the crystal violet bound to the biofilm. Finally, take an appropriate amount of decolorization solution and measure the absorbance of each well using an enzyme-linked immunosorbent assay (ELISA) reader at a wavelength of 570 nm. By comparing the OD values of the intervention group and the blank control group, the biofilm degradation rate of each group can be calculated using the formula "(control group OD value - experimental

group OD value)/control group OD value $\times$ 100%", thereby quantitatively evaluating their ability to remove biofilms.

4. Molecular mechanism of resveratrol in treating bone defects

4.1. Bioinformatics analysis of molecular mechanisms

To construct a multi omics mechanism map for the treatment of infectious bone defects with resveratrol, we conducted bioinformatics analysis using the following system process: Firstly, we searched and obtained disease-related genes in the Genecards database using keywords "bone defect", "bone infection", and "osteomyelitis", and selected genes with a Relevance score >10 to form a disease target set. After taking the intersection of the three, we obtained the core bone defect related targets [21]. Subsequently, the potential targets of resveratrol were comprehensively collected by integrating the ITCM Traditional Chinese Medicine Database and SwissTarget Prediction platform. The ITCM database searches for resveratrol keywords, and the SwissTarget Prediction platform uses the SMILES number of resveratrol, which can be retrieved from the PUBCHEM database for predicting the target [22]. Intersection of drug targets and disease targets to obtain a common target set. Import the collection into the STRING database (version 12.0), set the minimum confidence threshold for interactions to 0.7, construct a protein-protein interaction network, export the TSV format file, and visualize it using Cytoscape software (version 3.10.0) [23]. Further use Cytoscape's built-in Network Analyzer tool for topology analysis, based on the comprehensive scores of the three core parameters of "degree value", "betweenness centrality", and "proximity centrality", to screen the top 20 core targets [24]. Finally, these 20 core targets were submitted to the DAVID Bioinformatics Resource Platform (version 2021) for gene ontology (GO) functional enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, with a P-value <0.05 and a false discovery rate (FDR) <0.05 set as significant enrichment criteria to systematically elucidate their potential biological processes and signaling pathways.

4.2. Molecular docking verification

We obtained the three-dimensional structures of the target protein and resveratrol from RCSB PDB and PubChem databases, respectively, and used PyMOL and AutoDock Tools for pre-treatment such as water molecule removal, hydrogenation, and force field distribution. To comprehensively explore the possibility of binding, we adopt two complementary molecular docking strategies: first, we use AutoDock Vina for semi flexible docking within a predetermined active pocket (exhaustion = 32). At the same time, blind docking of the entire protein range was performed using the CB-Dock2 server to discover potential unknown sites. Finally, by comparing the results of the two methods comprehensively, the optimal binding mode is selected based on the principle of minimizing the binding free energy (ΔG , in kcal/mol), and its interaction is analyzed [25,26].

5. Design of animal experiments

5.1. Animal feeding and grouping

Healthy male SD rats aged 8-10 weeks were selected and fed with standard feed in SPF grade environment, with free access to water. After one week of adaptive feeding, they were used for the experiment. All animal operations follow the guidelines of the Animal Ethics Committee. The work has been reported in accordance with the ARRIVE guidelines (Animals in Research: Reporting In Vivo Experiments) [26-28]. Surgical instruments (drills, forceps, etc.) are sterilized by high-pressure steam; Disinfect the surgical area according to standard aseptic operating procedures; All experimental groups were directly used after rehydration with sterile PBS before microsphere implantation. The experimental

protocol was ethically approved by the Experimental Animal Welfare Ethics Committee of Southwest Medical University, approval No. 20231030-006 in its approval.

5.1.1. Construction of animal model for infectious bone defects

Referring to the mature methods of our team in the early stage, we constructed a bone defect model of rat femur and injected the MRSA model locally to infect the environment. Specifically, under sterile anesthesia conditions, a through bone defect with a diameter of approximately 3 mm is created at the femoral head using a ring drill or micro drill. After successful modeling, immediately use a microsyringe to accurately inject 10 μ L of methicillin-resistant *Staphylococcus aureus* containing 1×10^8 CFU into the defect cavity. Leave it for 5 min and then suture the wound to establish an infectious bone defect model.

5.1.2. Grouping of animals

The successfully modeled rats were randomly divided into the following four groups ($n = 10$):

Control group: Bone defect + MRSA infection model, without any drug intervention in the defect area.

GM group: Implant blank gelatin microspheres in the infected area of bone defects.

GM@RES Group: Implant gelatin microspheres loaded with resveratrol in the infected area of bone defects.

GM@RES@VAN group: Implant dual drug gelatin microspheres loaded with resveratrol and vancomycin in the infected area of bone defects.

5.2. Detection based on local bone tissue

Animals were euthanized at the fourth week after surgery, and bone tissue samples containing the defect area were taken. After fixation with 4% paraformaldehyde, decalcification, paraffin embedding, and sectioning were performed. The selection of 4 weeks as the observation endpoint is based on the following considerations: (1) In the rat bone defect model, 2-4 weeks after surgery is the active period of new bone formation, and at 4 weeks, the differences in bone repair between the groups have become statistically significant; (2) In a rat model of implant related osteomyelitis, 4 weeks is a commonly used endpoint for evaluating infection control and bone destruction progression; (3) The study on the treatment of multiple infectious bone defects with microspheres used a 4-week observation period, which is sufficient to evaluate the early bone regeneration effect [29–32].

5.2.1. H&E staining

Stain bone tissue sections using the hematoxylin eosin staining kit. Observe and evaluate the infiltration of inflammatory cells (such as neutrophils and lymphocytes), granulation tissue formation, and overall morphology of bone repair in the defect area through an optical microscope.

5.2.2. Masson staining

Perform Masson trichrome staining on bone tissue slices. After staining, collagen fibers appear blue, while muscle fibers and cytoplasm appear red. Evaluate the percentage of collagen deposition area in newly formed bone tissue in the defect area through image analysis software to assess bone matrix synthesis and maturity.

5.2.3. Galanz dyeing

Perform Gram staining on bone tissue slices. Observe and count the number of Gram positive cocci stained blue purple in the field of view under an oil microscope, and semi quantitatively evaluate the local residual MRSA bacterial load.

5.2.4. Detection of inflammatory factors

Detect local inflammation levels through immunofluorescence

technology. Paraffin sections of bone tissue were taken, and after antigen repair, mouse primary antibodies against rat IL-1 β , TNF- α , and IL-6 were added dropwise and incubated overnight at 4 °C. The next day, the sheep anti mouse IgG secondary antibody labeled with Cy3 was incubated in the dark, and the cell nucleus was counterstained with DAPI. Under a confocal laser microscope, the expression levels of key pro-inflammatory factors in each group were semi quantitatively analyzed by counting the intensity of specific fluorescent signals (red) per unit area.

5.3. Detection of osteogenic morphology and indicators

Collect ex vivo bone specimens and perform high-resolution scanning using a Micro CT scanner. Perform 3D reconstruction using supporting software and quantitatively analyze the following parameters of the bone defect area: Bone volume, Bone volume fraction, Trabecular number, Bone mineral density to accurately evaluate new bone formation and bone microstructure.

Extract new bone tissue from the defect area, extract total RNA, and reverse transcribe it into cDNA. Primers were designed based on the core target genes ALP, OPN, OCN, and COL I identified through bioinformatics analysis in the early and late stages of osteogenesis. GAPDH was used as an internal reference to detect the mRNA expression levels of these key genes in each group using real-time fluorescence quantitative PCR technology. The osteogenic mechanism of the drug was verified at the molecular level.

6. In vitro experimental verification GM@RES@VAN antibacterial osteogenic molecular mechanism

6.1. GM@RES@VAN cell toxicity and biocompatibility

The MC3T3-E1 mouse osteoblast cell line was chosen for the study, and normal grown MC3T3-E1 mouse osteoblast cells served as the untreated control group. The CCK-8 method was used to detect cell toxicity by co-culturing cells with varying concentrations of GM@RES@VAN microsphere extraction solution. The CCK-8 reagent was added at pre-determined time points (24, 48 h), incubated for 2 h, and the absorbance at 450 nm was measured using an enzyme-linked immunosorbent assay (ELISA) reader to calculate the cell proliferation efficiency.

Live dead staining of osteoblasts: Firstly, replace the original cell culture medium with serum-free working solution containing a specific concentration of dye, and incubate at 37 °C in the dark. After staining, observed under a fluorescence microscope: esterases in living cells hydrolyze non fluorescent Calcein AM into substances that emit strong green fluorescence. PI can only enter dead cells with damaged cell membranes and bind with nuclear DNA to produce red fluorescence. Evaluate the value-added effect of different materials on osteoblasts by comparing the green fluorescence intensity and cell count of different groups for three consecutive days.

Evaluate biocompatibility through ghost pen peptide staining: After fixing the cells, use ghost pen peptide (labeled F-actin) and DAPI (labeled nucleus) for staining, observe the morphology, spreading area, and adhesion of the cell skeleton under a microscope, and comprehensively evaluate the effect of the material on the normal morphology and function of the cells.

6.2. Osteogenic and vascular ability testing

6.2.1. Experimental design

Establishing an in vitro infection model: MC3T3-E1 cells were co cultured with MRSA in a certain ratio (e.g. 100:1) for 2 h to create a bacterial infection environment. The experiment was divided into three groups: control group (osteoblasts + bacterial infection), GM group (infection environment + blank gelatin microspheres) GM@RES@VAN group (infection environment + dual drug microspheres).

6.2.2. Evaluation of osteogenic differentiation function

Alkaline phosphatase (ALP) staining and activity quantification: BCIP/NBT substrates were used to qualitatively observe ALP staining following a 7-day osteogenic induction culture; Concurrently, the early osteogenic differentiation level was assessed by standardizing the total protein content, and the absorbance at 405 nm was quantitatively detected using an ALP kit. Evaluation of Late Mineralization Capability Using Alizarin Red S Staining: Cells were frozen and stained with 2% Alizarin Red S (pH 4.2) following 21 days of osteogenic induction in order to qualitatively assess the development of calcium nodules; After dissolving the nodules in a 10% hexadecylpyridine chloride solution, quantify the absorbance at 562 nm.

Similarly, primers were designed for the key indicators ALP, OPN, OCN, and COL I in the early and late stages of osteogenesis, with GAPDH as the internal reference. The mRNA expression levels of these key genes in each group were detected by real-time fluorescence quantitative PCR technology to verify the osteogenic mechanism of the drug at the molecular level.

6.2.3. Detection of differentiation of angiogenic ability

Using vascular endothelial cells as the research object, a bacterial infection environment was constructed by co culturing with MRSA in a certain ratio (such as 100:1) for 2 h. Grouping is consistent with osteogenic examination. Inoculate endothelial cells into a 24 well plate containing cover glass, intervene and fix with 4% paraformaldehyde for 15 min. Subsequently, the cells were permeabilized with 0.1% Triton X-100 for 10 min and blocked with 5% BSA for 1 h. Discard the blocking solution and directly add primary antibodies targeting endothelial markers (such as CD31), incubate overnight at 4 °C. The next day, after washing with PBST, Cy3 labeled secondary antibody was added and incubated at room temperature in the dark for 1 h. After washing again, stain the nuclei with DAPI for 5 min. Finally, seal the cover glass and observe it under a fluorescence microscope. Perform relative quantitative analysis of cell count by counting DAPI blue cell nuclei.

6.2.4. Key osteogenic and inflammatory protein detection

Based on the core signaling pathways screened through previous bioinformatics analysis, the expression of key proteins was detected by Western blot technology. Collect total protein from each group of cells, measure the concentration, and perform SDS-PAGE electrophoresis, membrane transfer, and blocking. Then, use specific primary antibodies (inflammation related proteins TNF- α , IL1B, IL6, JUN, key proteins for hypoxia, apoptosis, and autophagy BCL2, HIF1A, TP53, PTGS2, MMP9, etc.) to incubate overnight at 4 °C. After incubation with HRP labeled secondary antibodies at room temperature, analyze the relative expression level of the target protein by chemiluminescence imaging using GAPDH as an internal reference.

6.2.5. Observation of ultrastructure

Observation of mitochondrial morphology through transmission electron microscopy: Collect cells from each group, fix them with 2.5% glutaraldehyde, fix them with osmium acid, dehydrate them with acetone gradient, and embed them in epoxy resin to prepare ultra-thin sections. After double staining with uranium acetate and lead citrate, observe the morphological changes of cell mitochondria under transmission electron microscopy.

7. In vitro cell experiment design and HIF-1 α inhibition validation

To verify whether the effects of resveratrol depend on the HIF-1 α signaling pathway, the osteoblast cell line MC3T3-E1 was used for in vitro experiments. The model construction and drug administration were performed as described above. A total of eight experimental groups were set up:

Blank group (Blank): Conventional cultivation, Normal culture

without any treatment.

Model control group (Control): MC3T3-E1 cells were co-cultured with MRSA at a certain ratio (e.g., 100:1) for 2 h to establish a bacterial infection environment.

Blank microsphere group (GM): Model group + blank gelatin microspheres.

RES microsphere group (GM@RES): Model group + resveratrol-loaded microspheres.

VAN microsphere group (GM@VAN): Model group + vancomycin-loaded microspheres.

Dual-drug microsphere group (GM@RES@VAN): Model group + vancomycin and resveratrol co-loaded microspheres.

RES microsphere + HIF inhibitor group (GM@RES + 2-ME): Before adding the model stimulus and RES microspheres, cells were pretreated with the HIF-1 α specific inhibitor 2-methoxyestradiol (2-ME, 10 μ M, Sigma) for 2 h; the remaining procedures were the same as those for the RES-GM group.

Dual-drug microsphere + HIF inhibitor group (GM@RES@VAN + 2-ME): Cells were pretreated with 2-ME in the same manner, and the remaining procedures were the same as those for the VAN + RES-GM group.

After co-culturing all microspheres with cells for 24 h, cell lysates were collected, and the expression of the following nine proteins was detected by Western blot: HIF1A, IL1B, TNF- α , IL6, BCL2, JUN, p53, MMP9, and PTGS2. GAPDH was used as an internal control. By comparing protein expression differences with or without 2-ME pretreatment, the causal necessity of the HIF-1 α pathway in resveratrol-mediated anti-inflammatory, anti-apoptotic, and matrix-regulating effects was validated. Each group was performed with three independent replicates, and each replicate contained three technical replicates.

8. Materials and reagents

The main materials and reagents used in this study are detailed as follows: Methacrylated gelatin (GelMA, substitution degree ~60%, catalog No. G477882, Aladdin, Shanghai, China); photoinitiator sodium phenyl(2,4,6-trimethylbenzoyl)phosphinate (NAP, purity $\geq 99.5\%$, molecular weight 242.1 Da, catalog No. P1518380, Aladdin, Shanghai, China); resveratrol (RES, purity $\geq 99\%$, molecular weight 234.20 Da, catalog No. R1418232, Aladdin, Shanghai, China); vancomycin hydrochloride (VAN, potency ≥ 900 μ g/mg, molecular weight 1485.71 Da, catalog No. V432044, Aladdin, Shanghai, China); liquid paraffin (chemical grade, boiling range 300–500 °C, density 0.835–0.895 g/mL, catalog No. P104805, Aladdin, Shanghai, China); sorbitan monooleate (Span-80, HLB value 4.3, catalog No. T196269, Aladdin, Shanghai, China); hyaluronidase (from bovine testes, activity 300–1000 U/mg, catalog No. H754887, Aladdin, Shanghai, China); dialysis bag (molecular weight cut-off 3500 Da, flat width 18 mm, Aladdin, Shanghai, China). The methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC 43300) used for antibacterial assays was purchased from the American Type Culture Collection (ATCC). The bacterial live/dead staining was performed using a Calcein-AM/PI double staining kit (catalog No. A1492198, Aladdin, Shanghai, China). Crystal violet staining kit (catalog No. C1506003, Aladdin, Shanghai, China). Bone tissue staining: hematoxylin-eosin (HE) staining kit (catalog No. H1506002, Aladdin, Shanghai, China); Masson's trichrome staining kit (catalog No. M774209, Aladdin, Shanghai, China); Gram staining kit (catalog No. S774843, Aladdin, Shanghai, China). Primary antibodies for immunofluorescence: mouse anti-rat IL-1 β (catalog No. ab255118, Abcam, UK), TNF- α (catalog No. ab1793, Abcam), IL-6 (catalog No. ab9324, Abcam); secondary antibody: Cy3-labeled goat anti-mouse IgG (catalog No. A10520, Invitrogen, USA); nuclear dye DAPI (catalog No. D9542, Sigma, USA). The MC3T3-E1 mouse pre-osteoblast cell line and HUVEC human umbilical vein endothelial cells used in cell experiments were purchased from Shanghai, China. CCK-8 kit (catalog No. C0037, Beyotime, China); assay conditions: 10 μ L of CCK-8 solution was added to each well,

incubated at 37 °C for 2 h, and the absorbance was measured at 450 nm. Phalloidin-iFluor 488 (catalog No. ab176753, Abcam, UK) staining conditions: after fixation, cells were permeabilized with 0.1% Triton X-100, blocked with 5% BSA, and then incubated with 1:200 diluted phalloidin for 1 h at room temperature in the dark. Alkaline phosphatase (ALP) staining kit (BCIP/NBT method, catalog No. C3206, Beyotime) and ALP activity quantitative assay kit (catalog No. P0321S, Beyotime). For the quantitative ALP assay, p-nitrophenyl phosphate (pNPP) was used as substrate; the reaction was carried out at 37 °C for 30 min, and the absorbance was measured at 405 nm, then normalized to total protein content determined by BCA assay. Alizarin Red S (catalog No. A5533, Sigma, USA). Primary antibodies for Western blot: TNF- α (#11948), IL-1 β (#12703), IL-6 (#12912), JUN (#9165), BCL2 (#3498), HIF1A (#36169), TP53 (#2524), PTGS2 (#12282), MMP9 (#13667) and GAPDH (#5174) were all purchased from Cell Signaling Technology (USA); HRP-labeled goat anti-rabbit secondary antibody (#7074, CST); chemiluminescent substrate (Thermo Fisher, USA). HIF-1 α specific inhibitor 2-methoxyestradiol (2-ME, catalog No. M3329, Sigma, USA) was used at a concentration of 10 μ M with a pretreatment time of 2 h. Centrifugation conditions: 1000–1500 g, 3–5 min, room temperature. Freeze-drying conditions: temperature –50 °C, vacuum 10 Pa, duration 24 h. All assays were performed according to the manufacturers' instructions, and the laboratory temperature was maintained at 22–25 °C (except for specific incubations at 37 °C).

9. Statistical analysis

All quantitative data in this study were presented as mean $\pm$ standard

deviation and analyzed using GraphPad Prism 10.0 software. In vitro cell experiments were conducted using one-way ANOVA for multiple group comparisons, and Tukey's test was used for pairwise comparisons between groups; In vivo animal experiments were conducted using two factor analysis of variance to evaluate the main effects and interactions between material treatment and genotype. If the interaction was significant, a simple effects analysis was performed. Non parametric tests are used for non normally distributed data, and Pearson or Spearman methods are used for correlation analysis depending on the distribution of the data. All experimental designs were determined by efficacy analysis to ensure a sample size of ≥ 0.8 and a significance level of $\alpha = 0.05$.

10. Results

10.1. Characterization results of microspheres

Electron and scanning electron microscopy observations demonstrate that GM@RES@VAN composite drug-loaded microspheres have been successfully manufactured using microfluidic technology (Fig. 1). The microspheres appear as regular spherical shapes with smooth surfaces, good dispersion, and no discernible adhesion when seen under an electron microscope. The SEM image is used to observe the overall morphology of the microspheres, the uniformity of particle size, and any potential internal interfaces - small particles with attached drugs, especially at a ratio of 5.0 μ m (Fig. 2A). ImageJ statistical analysis shows that the diameter distribution of microspheres is uniform, with an average diameter concentrated in the range of 230–250 μ m (Fig. 2B and

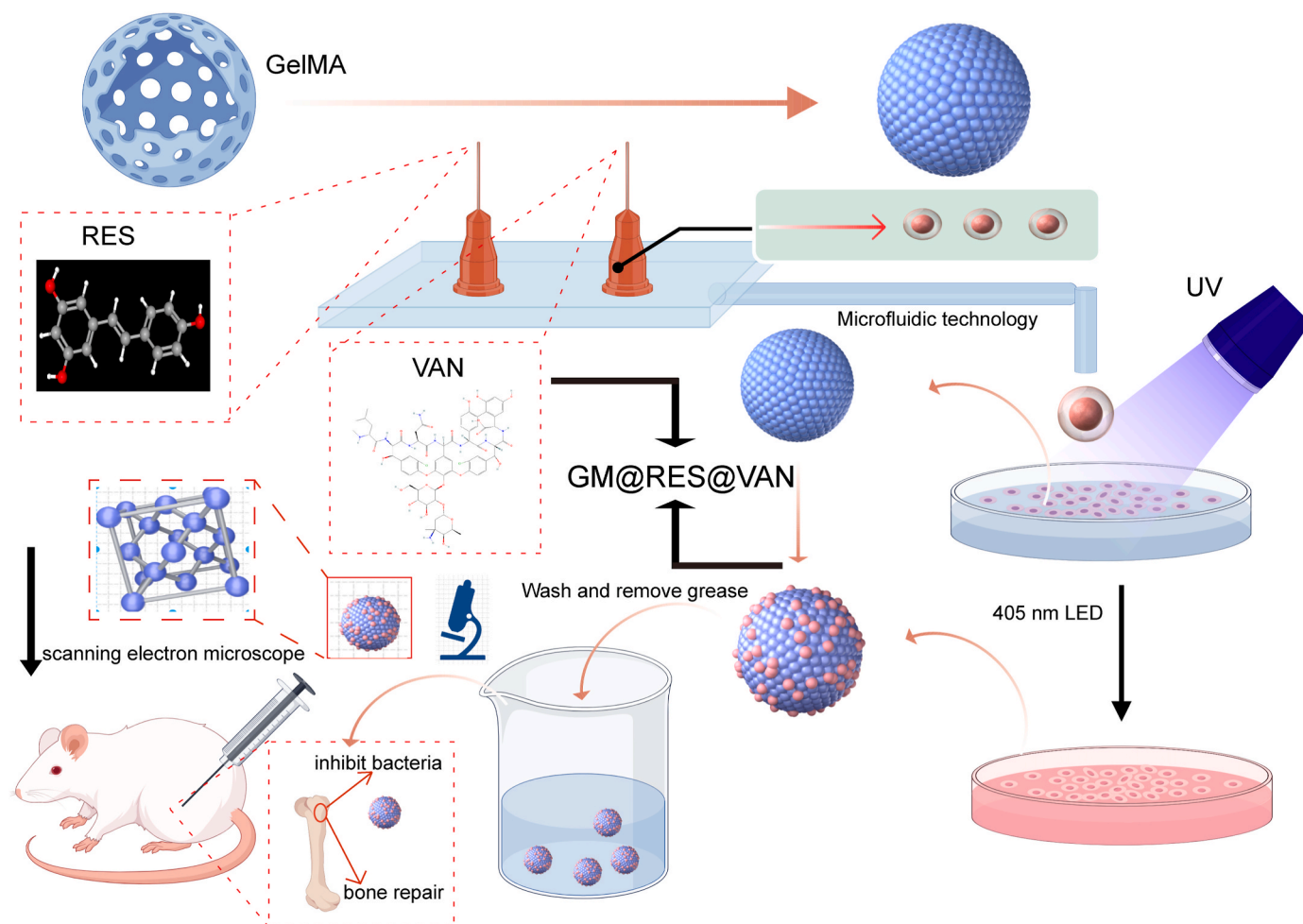
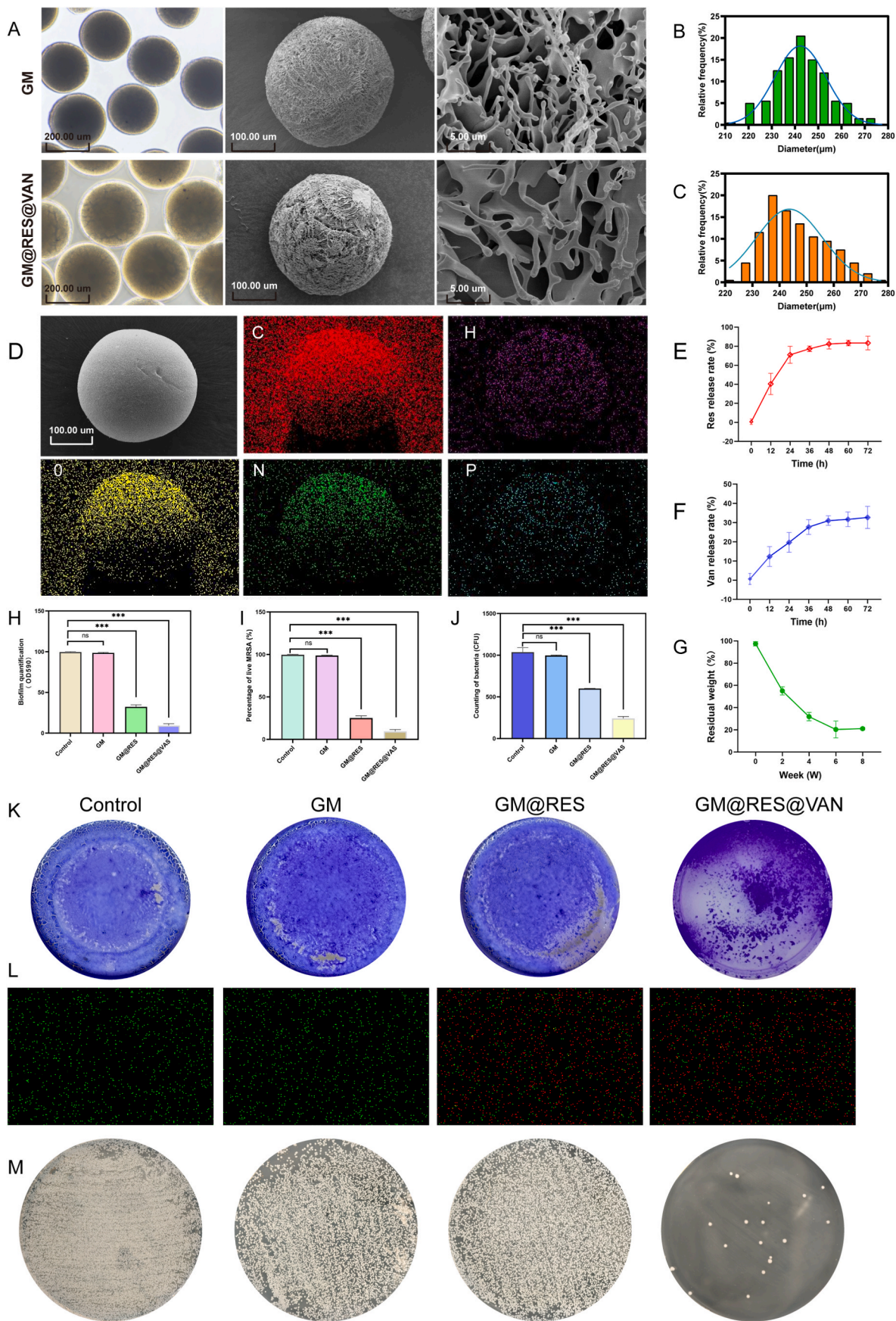


Fig. 1. Schematic diagram of the microfluidic technology for microsphere fabrication and local rat injection.



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Fig. 2. Characterization of Microsphere Structure and Antibacterial Experiment: A. Characterization of Blank Microspheres and Drug loaded Microspheres in the 200–100–5 μm Scale. B. The diameter distribution histogram of blank microspheres, with an average diameter of 230–250 μm . C. The diameter distribution histogram of drug loaded microspheres, with an average diameter of 230–250 μm . D. The characteristic elements of drug loaded microspheres are carbon (C), hydrogen (H), oxygen (O), nitrogen (N), and phosphorus (P) distribution. E. The 72 h drug release curve of resveratrol shows a sustained release of approximately $82.33 \pm 2.08\%$ within 48 h. F. The 72 h drug release curve of vancomycin achieved a sustained release of $31.00 \pm 1.0\%$ within 48 h. G. The 8-week degradation curve of drug loaded microspheres decreased to a minimum of about 20% in the 6th week. H. Statistical analysis bar chart of OD590 values of bacterial biofilm in different groups, the OD590 value of the GM@RES@TVAN treatment group was significantly lower than that of the Control groups ($n = 3$, $***p < 0.001$). I. The proportion of viable bacteria in different groups, The bacterial survival rate of the GM@RES@VAN group was significantly lower than that of the Control groups ($n = 3$, $***p < 0.001$). J. Bar charts of bacterial counts in different groups. The inhibition rate of GM@RES@VAN group was significantly higher than that of Control groups ($n = 3$, $***p < 0.001$). K. Photographs of bacterial biofilms in different groups. L. Photographs of bacterial viability staining in different groups. M. Photographs of bacterial counts in different groups.

C). Elemental analysis using an energy spectrometer can detect characteristic elements such as carbon (C), hydrogen (H), and oxygen (O) that may exist in the core region of vancomycin and resveratrol molecules. In addition, nitrogen (N) and phosphorus (P) are common basic elements in organic compounds and have also been detected, which intuitively proves that the two drugs have been successfully loaded inside the microspheres (Fig. 2D). Resveratrol maintains a sustained release of approximately $82.33 \pm 2.08\%$ within the first 48 h, but gradually stabilizes thereafter, and this release pattern is highly consistent with bone repair needs (Fig. 2E). At the same time, the release of vancomycin reached a sustained release of $31.00 \pm 1.0\%$ in the first 48 h, which is equivalent to the clinical application of bone cement releasing approximately 10%–30% of the total drug loading within 72 h (Fig. 2F). The sustained release curve of the drug shows that GM coating technology significantly improves the drug loading efficiency of bone repair materials, while reducing the drug release rate. This not only prolongs the treatment period but also effectively inhibits chronic inflammation. Finally, the remaining weight percentage of microspheres showed a decrease over time, reaching a minimum of approximately 20% at week 6 (Fig. 2G).

10.2. GM@RES@VAN antibacterial effect

The biofilm degradation experiment further showed that, GM@RES@VAN, the OD590 value of the treatment group was significantly lower than that of the other groups ($n = 3$, $***p < 0.001$), and the calculated biofilm degradation rate was the highest, indicating that the dual drug microspheres can not only kill planktonic bacteria, but also effectively destroy the formed MRSA biofilm (Fig. 2H–K). The live and dead staining results of bacteria showed that under the fluorescence microscope, the fields of view of the control group and GM group were mainly green fluorescence (live bacteria), while large areas of red fluorescence (dead bacteria) appeared in the field of view of the GM@RES@VAN group. Quantitative analysis shows that, GM@RES@VAN, the bacterial survival rate of the group was significantly lower than that of the other groups ($n = 3$, $***p < 0.001$) (Fig. 2I–L). The bacterial coating experiment showed that compared with the control group and GM group, GM@RES group showed slight antibacterial activity, while GM@RES@VAN group showed the strongest antibacterial effect. The colony counting results showed that, the inhibition rate of GM@RES@VAN group was significantly higher than that of other groups ($n = 3$, $***p < 0.001$), approaching complete bacterial clearance (Fig. 2J–M).

10.3. Molecular mechanism of resveratrol in treating bone defects

We have found 1479 disease targets associated with bone abnormalities through keyword search and data intersection. Following that, 143 resveratrol pharmacological targets were gathered using molecular prediction platforms and databases from traditional Chinese medicine. Based on the intersection of medications and disorders, 55 possible therapeutic targets were ultimately identified (Supplementary Tables S1–S5, Fig. 3A). A network of 55 protein interactions was created using STRING, demonstrating that the majority of proteins

performed well and were connected. We created a network of 20 core targets for resveratrol treatment of bone abnormalities by using Dgree to sort in decreasing order. IL6, JUN, TNF, IL1B, MMP9, CCL2, PTGS2, BCL2, TP53, MAPK3, VCAM1, SRC, EGFR. It may play a key role in the formation and treatment of bone defects (Fig. 3B and C). The biological processes suggested in GO enrichment analysis are mainly related to carbon metabolism, cell response to lipopolysaccharides, peptide tyrosine autophosphorylation, platelet activation, positive regulation of interleukin-8 production, positive regulation of phosphatidylinositol 3-kinase/protein kinase B signaling transduction, response to exogenous stimuli, cell response to reactive oxygen species, negative regulation of apoptosis process, and positive regulation of apoptosis process. The main cellular components involved include extracellular space, membrane raft, cytoplasm, mitochondria, exogenous components on the cytoplasmic side of the plasma membrane, extracellular regions, and adhesive plaques. Its molecular functions mainly involve carbonate removal, hydrolase activity, heme binding, enzyme binding, protein tyrosine kinase activity, zinc ion binding, phospholipase binding, general transcription start factor binding, protein homodimerization activity, and SH2 domain binding (Supplementary Table S6, Fig. 3D and E). Finally, based on the KEGG signaling pathway, there is a significant correlation between apoptosis and autophagy in cellular processes, with the main environmental signaling pathways being the TNF and HIF signaling pathways. Finally, we can also observe the differentiation of osteoclasts involved.

After initially screening 20 core targets through network pharmacology, we further selected 9 key targets for Western blot validation based on KEGG enrichment analysis, target interaction network rankings, and the pathological characteristics of infected bone defects. Enrichment analysis revealed that the 20 core targets were significantly enriched in the apoptosis pathway, TNF signaling pathway, and HIF-1 signaling pathway. These three pathways correspond respectively to the two fundamental pathological mechanisms of infected bone defects: inflammatory response (TNF pathway) and local hypoxia (HIF-1 pathway), while the apoptosis pathway represents the downstream common outcome of both. Accordingly, we selected representative targets with high rankings and well-defined functions from each pathway: inflammation-related targets IL1B, TNF, and IL6; the master regulator of hypoxia, HIF1A; and apoptosis-related targets BCL2 (anti-apoptotic) and JUN (pro-apoptotic). In addition, considering that three multi-functional targets—TP53, MMP9, and PTGS2—were among the top 10 in the interaction network and are involved in cell cycle regulation, matrix remodeling, and inflammatory mediator synthesis, respectively, which are closely associated with bone repair and the infection micro-environment, they were also included for detection. The final nine validated targets were: IL1B, TNF, IL6, HIF1A, BCL2, JUN, TP53, MMP9, and PTGS2. This selection strategy balanced pathway integrity, target priority, and pathological relevance (Supplementary Table S7, Fig. 3F and G).

The molecular docking results showed that resveratrol has strong binding activity with core target proteins, with binding free energies (ΔG) all < -5.0 kcal/mol, indicating that it can spontaneously and stably bind to the active pockets of these targets, theoretically verifying the possibility of resveratrol acting through these targets (Fig. 4A–I).

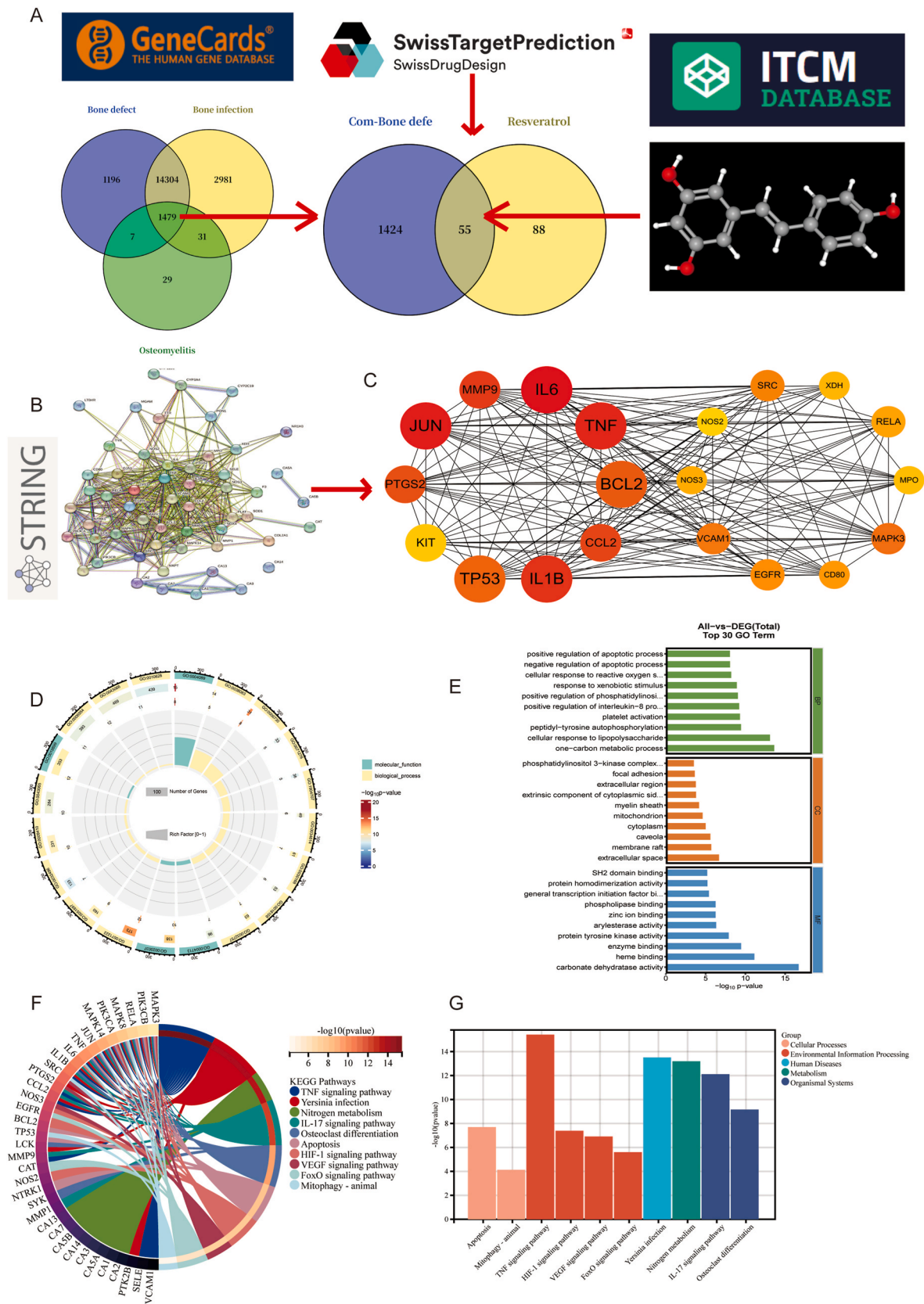


Fig. 3. Bioinformatics analysis of resveratrol treatment for bone defects: A. Screening of 55 proteins involved in resveratrol treatment for bone defects using GeneCards, ITCM, and Swiss Target Prediction. B. Protein interaction network diagram of proteins involved in resveratrol treatment for bone defects. C. 20 core targets of resveratrol treatment for bone defects, mainly involving inflammatory factors such as IL1B, TNF-a, and IL6. D. The GO enrichment analysis and chord plot of 20 core target genes showed that they were mainly related to cell response to lipopolysaccharides and peptide tyrosine autophosphorylation. E. The GO enrichment analysis bar chart of 20 core target genes. F. The KEGG enrichment analysis chord plot of 20 core target genes. G. The KEGG enrichment analysis bar chart of 20 target genes showed that they were closely related to apoptosis, TNF, and HIF signaling.

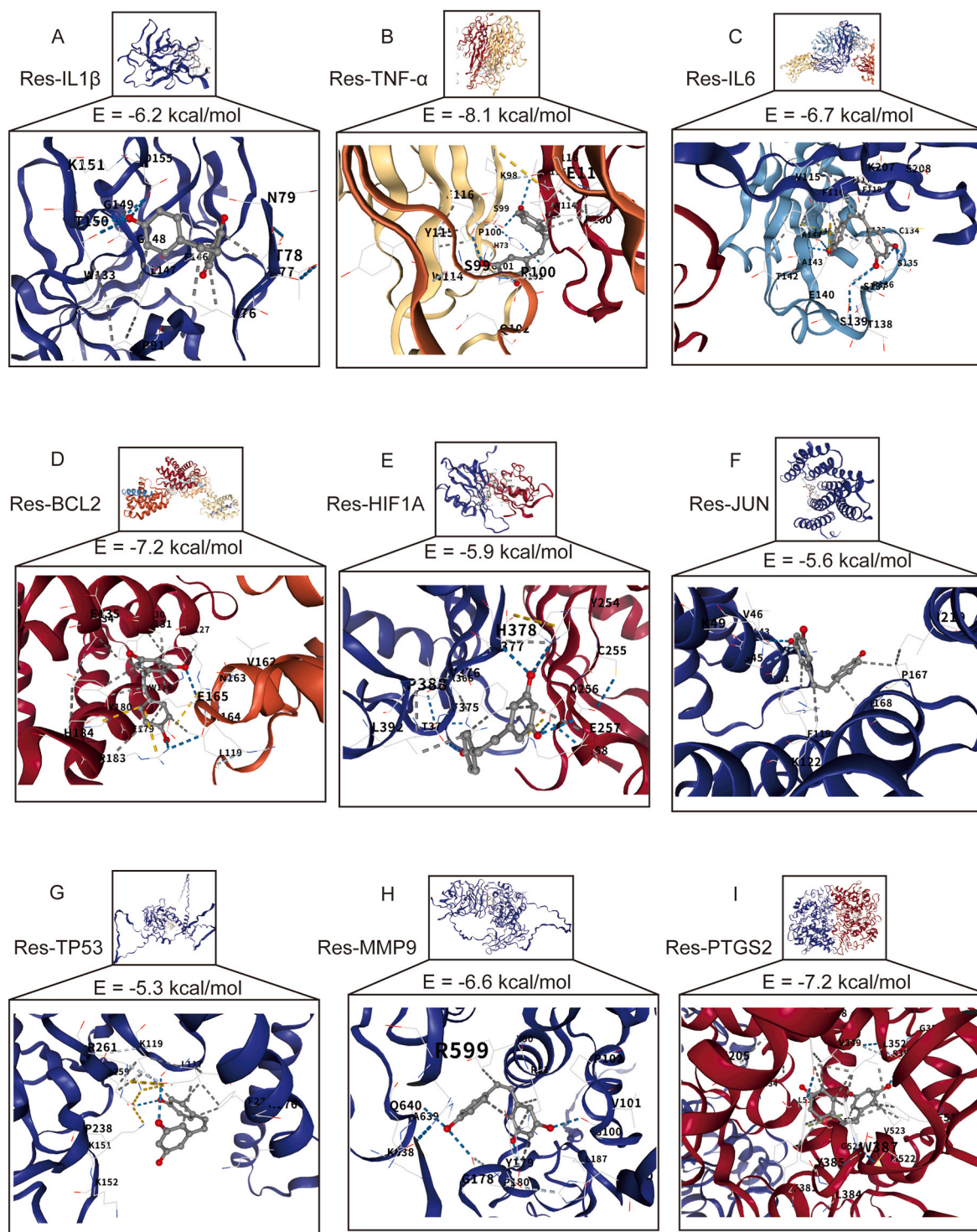


Fig. 4. Molecular Docking Diagram of Resveratrol and 9 Core Proteins: A. Docking Results of Resveratrol and IL1 β with Binding Energy $E = -6.2$ kcal/mol, B. Docking Results of Resveratrol and TNF- α with Binding Energy $E = -8.1$ kcal/mol, C. Docking Results of Resveratrol and IL6 with Binding Energy $E = -6.7$ kcal/mol, D. Docking Results of Resveratrol and BCL2 with Binding Energy $E = -7.2$ kcal/mol, E. Docking Results of Resveratrol and HIF1A with Binding Energy $E = -5.9$ kcal/mol, F. Docking Results of Resveratrol and JUN with Binding Energy $E = -5.6$ kcal/mol, G. Docking Results of Resveratrol and TP53, Combined Energy $E = -5.3$ kcal/mol, H. Resveratrol and MMP9 docking results, binding energy $E = -6.6$ kcal/mol, I. Resveratrol and PTGS2 docking results, binding energy $E = -7.2$ kcal/mol.

10.4. In vivo experimental results

HE staining showed that a large number of inflammatory cells infiltrated the defect area in the control group and GM group, with extensive granulation tissue formation and no obvious signs of bone

repair. The inflammation of the GM@RES group has been alleviated, and the proportion of inflammatory cells has decreased from 94.86% in the control group to 73.82%. But The infiltration of inflammatory cells in the GM@RES@VAN group was significantly reduced to 19.83%, indicating more orderly formation of new bone tissue and cartilage

callus. Meanwhile, the proportion of inflammatory cells in the GM@RES@VAN group was significantly lower than that in the GM@RES group ($n = 3$, $***p < 0.001$), indicating that the combined use of drugs yields better results than using RES alone. (Fig. 5A–D). Masson staining showed that the control group and GM group had less deposition of blue collagen fibers in the defect area, accounting for less than 20%. The deposition of collagen fibers in the GM@RES group increased relatively, reaching 43.20%. Among which, The GM@RES@VAN group showed the richest deposition of blue collagen fibers in the defect area, with a significantly higher area percentage than other groups, reaching 90.60%, indicating that the GM@RES@VAN group had the best synthesis and maturity of bone matrix. Meanwhile, The deposition of collagen fibers in GM@RES@VAN group was significantly higher than that in GM@RES Group ($n = 3$, $***p < 0.001$), indicating that dual drug microspheres are more effective than loading RES alone. (Fig. 5B–E). Gram staining showed a large number of blue purple Gram positive cocci in the field of view of the control group and GM group, with positive rates of 42.68% and 34.34%, respectively. A small amount of Gram positive cocci were also observed in the GM@RES group, In the GM@RES@VAN group, only sporadic or almost no bacteria were observed in the field of view, and the positivity rate decreased to 8.50%. The local bacterial load was significantly lower than in the other groups (Fig. 5C–F). Meanwhile, The bacterial positivity rate in the GM@RES@VAN group was significantly lower than that in the GM@RES Group ($n = 3$, $**p < 0.01$), indicating that dual drug microspheres have better antibacterial effects than RES loaded alone. In summary, the above results suggest that the dual drug microsphere group has better anti-inflammatory, collagen fiber formation promoting, and bacterial inhibition effects than the single drug (resveratrol) group, which may be due to the synergistic effect of vancomycin and resveratrol. On the one hand, resveratrol has significant anti-inflammatory properties and promotes collagen formation. On the other hand, the antibacterial effect of vancomycin clears obstacles for the action of resveratrol. The mutual promotion between the two enables the dual drug microspheres to play a better role.

The immunofluorescence results showed that IL-1 β , as a star factor of inflammation, was significantly expressed in the control group, while the level in the GM group was reduced. The red fluorescence signal of IL-1 β in bone tissue slices was significantly weakened in GM@RES and GM@RES@VAN, The GM@RES@VAN group has the weakest strength (Fig. 6A–D, $n = 3$, $***p < 0.001$). TNF - α and IL-6 showed a trend similar to IL-1 β in each group, and GM@RES and GM@RES@VAN, the intensity of the group's red fluorescence signal significantly decreased (Fig. 6B–C, $n = 3$, $***p < 0.001$). In summary, quantitative analysis indicates that GM@RES@VAN, the expression levels of pro-inflammatory factors in the group were significantly lower than those in the control group and GM group ($**p < 0.01$) (Fig. 6D–G). Meanwhile, all results indicate that, The inflammatory levels in the GM@RES@VAN group were significantly lower than those in the single drug microsphere group (GM@RES). This further indicates that the anti-inflammatory effect of the dual drug microspheres is significantly better than that of the single resveratrol microsphere group. This result once again confirms that the anti-inflammatory effect of dual drug microspheres is better ($n = 3$, $**p < 0.01$, $*p < 0.05$, red statistical line).

The three-dimensional reconstruction image visually displays a large number of shadows and broken, chaotic trabeculae around the coronal and sagittal nails in the control group, indicating the success of the bone defect model. The bone defect repair in the GM microsphere group was not significant, and chaotic bone trabeculae were still visible. GM@RES Preliminary display of repaired and formed bone tissue, with relatively neat arrangement of bone trabeculae. Finally, the repair of bone defects in the GM@RES@VAN group was the most complete (Fig. 7A). Quantitative analysis shows that, compare with the control group: The bone volume of the GM@RES@VAN group increased from 14.83 mm³ in the control group to 21.95 mm³. The percentage of bone volume/tissue volume also increased from 0.49 in the control group to the

GM@RES@VAN group has a score of 0.61. Bone mineral density increased from 283.53 mg/cm³ in the control group to 389.72 mg/cm³ in the GM@RES@VAN group. The number of bone trabeculae is also present GM@RES@VAN group significantly increased to 1.32 mm⁻¹. In summary, CT results indicate the Bone volume, bone volume/tissue volume, Bone mineral density, and number of bone trabeculae in the GM@RES@VAN group were significantly increased ($**p < 0.01$), indicating that it has the best new bone generation and bone microstructure (Supplementary Table S8, Fig. 7B–E). Although, all bone parameters in the GM@RES@VAN group are compared GM@RES group showed some improvement ($n = 3$ ns), but none of them were statistically significant, indicating that vancomycin has no practical significance in promoting osteogenesis, while resveratrol plays an important role in promoting osteogenesis. Vancomycin may exert an indirect effect by inhibiting bacterial growth. RNA detection of newly formed bone tissue in the defect area showed that, the anti apoptotic protein BCL2 was significantly expressed in the GM@RES@VAN group, while the hypoxia inducible factor HIF1A, extracellular matrix degrading MMP9, and inflammatory enzyme PTGS2 were significantly reduced. This indicates that the material may promote osteogenic repair by enhancing bone cell anti apoptosis, reducing hypoxia and inflammatory microenvironment, and improving extracellular matrix processes (Fig. 7F). This is consistent with the prediction results of bioinformatics, revealing the role of resveratrol in promoting osteogenesis and inhibiting inflammation in vivo at the molecular level. Finally, as key proteins involved in bone formation, ALP, OPN, OCN, and COL I are all present in the GM@RES@VAN group showed significant expression (Fig. 7G, $n = 3$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

10.5. In vitro experimental results

10.5.1. Cell toxicity and biocompatibility of GM@RES@VAN

Cell viability staining showed that, GM@RES group and GM@RES@VAN, the green fluorescence intensity of this group cells significantly increased on the 5th day, and the treatment group was significantly higher than the control group, indicating that the material does not cause significant oxidative stress damage and has good biocompatibility (Fig. 8A–D). In addition, the ghost pen cyclic peptide data showed that the co cultured cells of the two microsphere groups had good spreading, clear cell cytoskeleton, sufficient extension, and no difference in normal cell morphology, indicating that the material has good cell compatibility and does not affect cell adhesion and spreading (Fig. 8E and F). Different concentrations, after co culturing with GM@RES@VAN extraction solution, osteoblasts significantly increased in value at all time points, which was significantly different from the control group, indicating that the material had no significant cytotoxicity and could promote cell proliferation. The most significant proliferation effect was observed at 10 μ mol/L after 24 h (Fig. 8G and H).

10.5.2. Analysis of osteogenic mechanism of GM@RES@VAN

ALP staining and quantification: In an infectious environment, The GM@RES@VAN group had the deepest ALP staining, and quantitative results also showed that its ALP activity was significantly higher than that of the control group and the GM@RES group ($n = 3$, $**p < 0.01$) showed that resveratrol can effectively promote early differentiation of osteoblasts (Fig. 9A–C). Alizarin Red Staining: The GM@RES@VAN group formed the most calcium nodules and had the deepest staining. Quantitative analysis showed that its mineralized nodule content was significantly higher than other groups, proving that it can strongly promote late mineralization of osteoblasts (Fig. 9B–D). Angiogenesis experiment: GM@RES group, and the vascular formation in the GM@RES@VAN group was higher than that in the control group, but there was no significant difference between the two treatment groups, indicating that resveratrol has a certain ability to promote angiogenesis, but vancomycin has no related ability (Fig. 9E–J). Osteogenic indicator RNA detection: Similarly, for the key proteins of bone formation ALP,

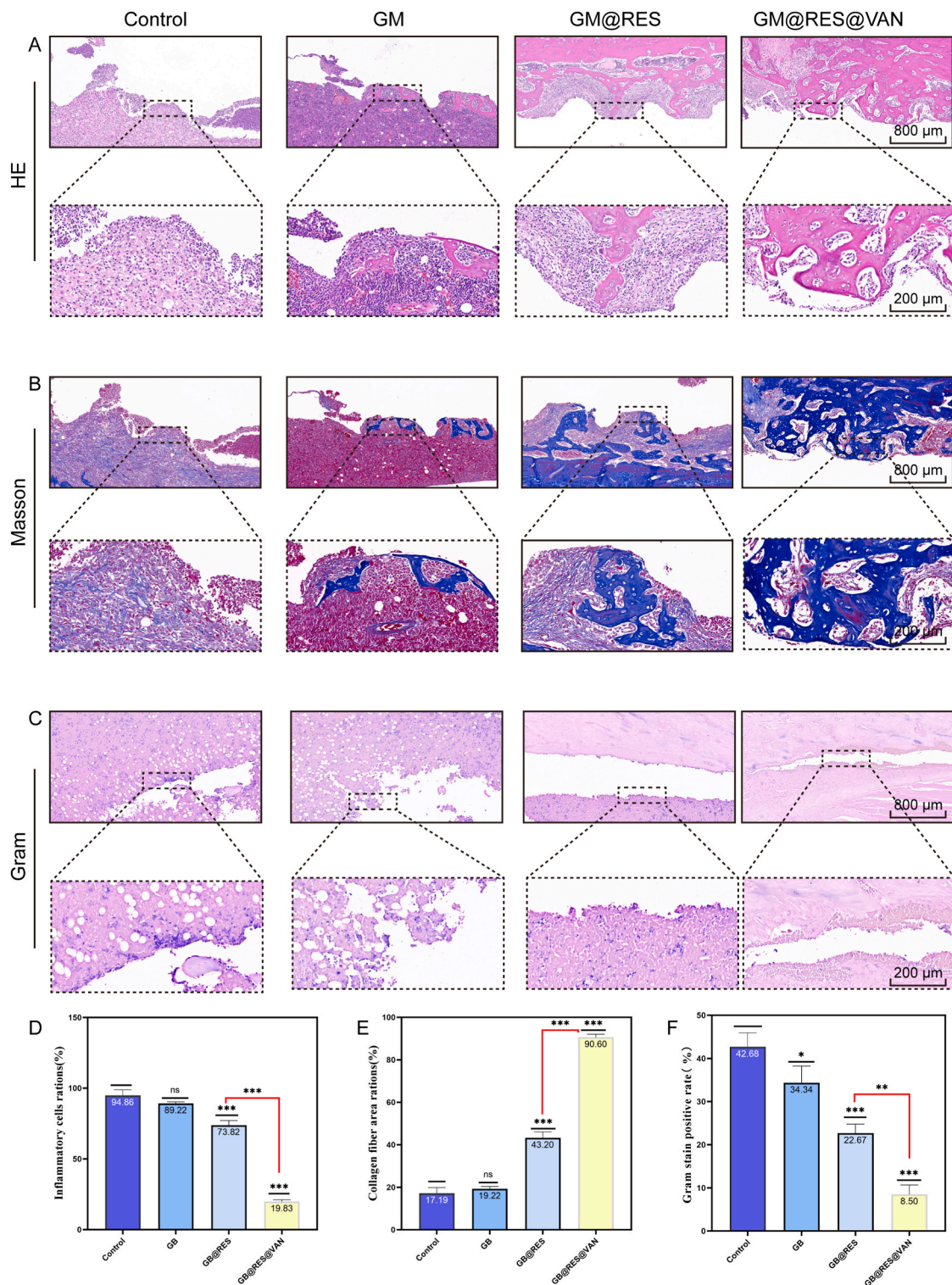


Fig. 5. Tissue staining of different groups: A-D. Compared with the control group, The inflammation of the GM@RES group has been alleviated, and the proportion of inflammatory cells has decreased to 73.82%. The infiltration of inflammatory cells in the GM@RES@VAN group was significantly reduced, decreasing to 19.83%. Meanwhile, The GM@RES@VAN group was significantly lower than GM@RES Group ($n = 3$, $***p < 0.001$). B-E. Compared with the control group, the GM group had less deposition of blue collagen fibers in the defect area. The deposition of collagen fibers in the GM@RES group increased relatively, reaching 43.20%. The GM@RES@VAN group showed the richest deposition of blue collagen fibers in the defect area, reaching 90.60%. Meanwhile, The GM@RES@VAN group was significantly higher than GM@RES Group ($n = 3$, $***p < 0.001$). C-F. Gram staining showed that compared with the control group, A small amount of Gram positive cocci were also observed in the GM@RES group, In the GM@RES@VAN group, only sporadic or almost no bacteria were observed in the field of view, and the positivity rate decreased to 8.50%. Meanwhile, The GM@RES@VAN group was significantly lower than GM@RES Group ($n = 3$, $**p < 0.01$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

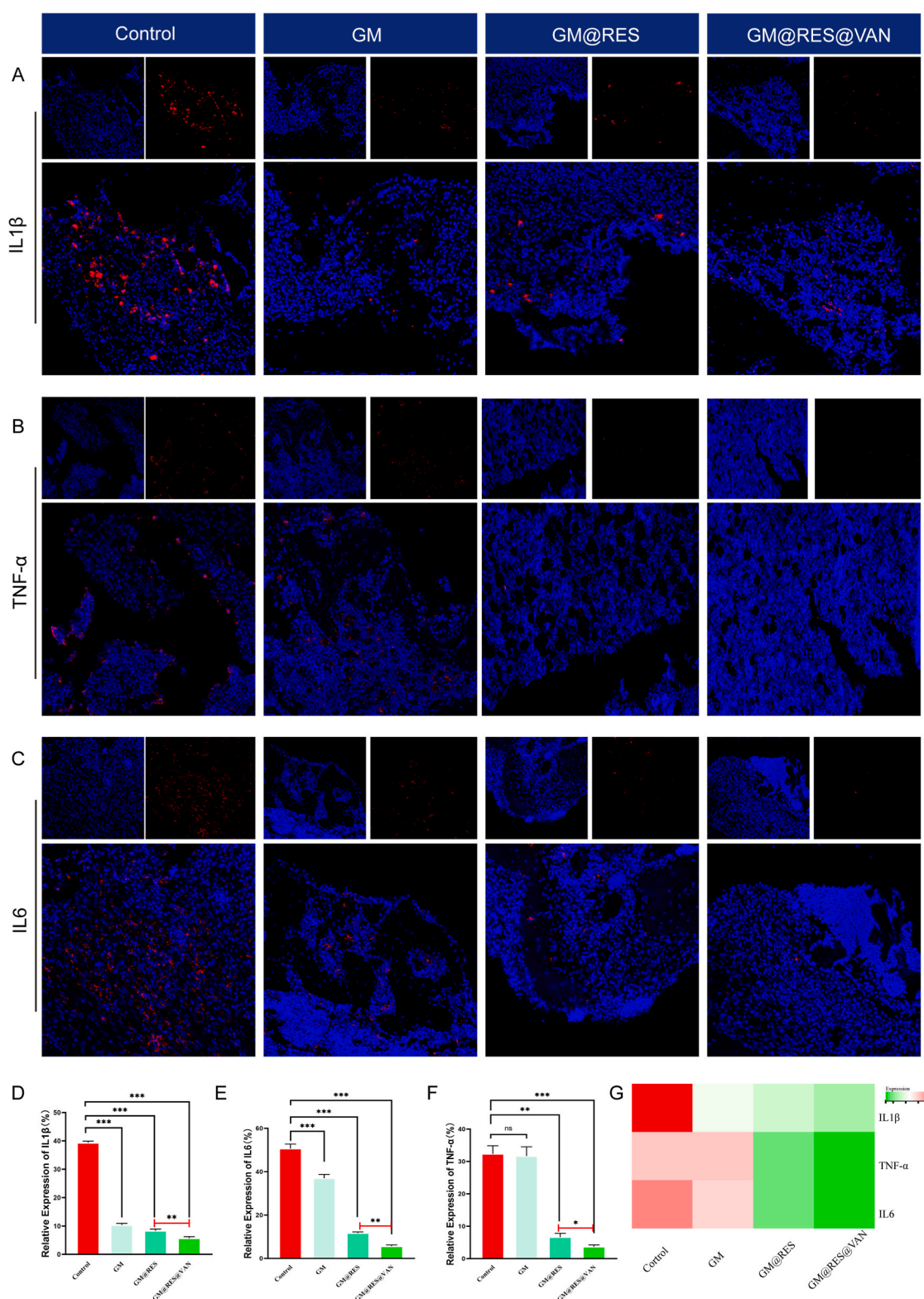


Fig. 6. Immunofluorescence detection of inflammatory factors: A-D. Compared with the control group, IL1 β was detected in the GM group, GM@RES Group, GM@RES@VAN group showed a significant decrease ($n = 3$, $***P < 0.001$). Meanwhile, GM@RES@VAN group comparison GM@RES Group significantly decreased ($n = 3$, $**P < 0.01$, red statistical line). B-E. Compared with the control group, TNF- α in the GM group, GM@RES Group and GM@RES@VAN group showed a significant decrease ($n = 3$, $***P < 0.001$). Meanwhile, GM@RES@VAN group comparison GM@RES Group significantly decreased ($n = 3$, $**P < 0.01$, red statistical line). C-F. It showed no significant difference in IL6 between the GM group and the control group ($n = 3$, ns), GM@RES Group ($n = 3$, $**P < 0.01$). The GM@RES@VAN group showed a significant decrease ($n = 3$, $***P < 0.001$). Meanwhile, GM@RES@VAN group comparison GM@RES Group significantly decreased ($n = 3$, $*P < 0.05$, red statistical line). G. Heat maps of the expression of IL1 β , TNF- α , and IL6 in different groups.

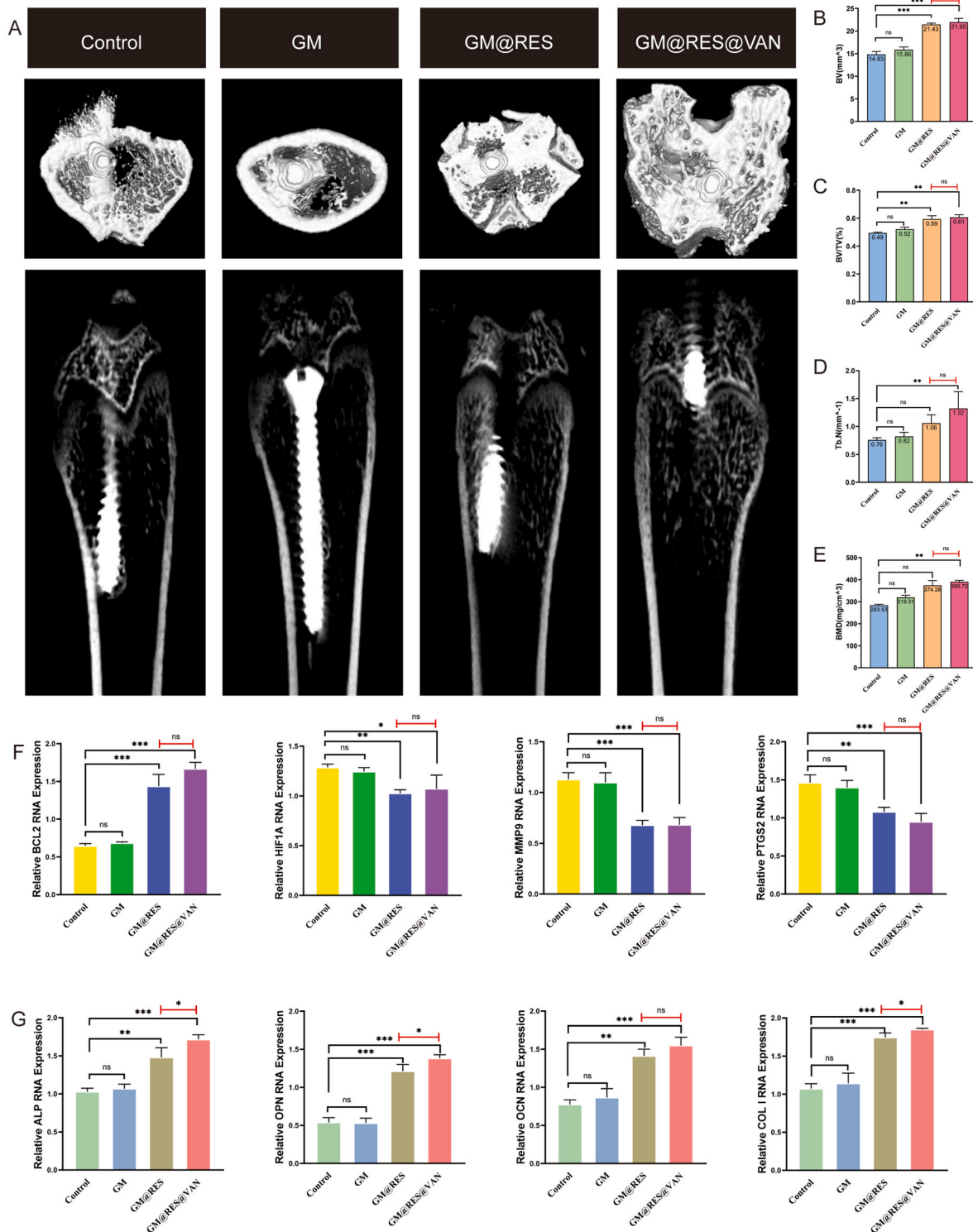


Fig. 7. A. CT sagittal and coronal results of bone tissue in different groups, B. Comparison with the control group, The BV of GM@RES and GM@RES@VAN group significantly increased, but GM@RES@VAN group comparison GM@RES, The group increased without statistical significance. C. Compared with the control group, The BV/TV of GM@RES and GM@RES@VAN group significantly increased, but GM@RES@VAN group comparison GM@RES, The group increased without statistical significance. D. The Tb.n of GM@RES@VAN group significantly increased, but GM@RES@VAN group comparison GM@RES, The group increased without statistical significance. E. The BMD of the GM@RES@VAN group significantly increased, but GM@RES@VAN group comparison GM@RES, The group increased without statistical significance. F. Compared with the control group, BCL2 was found to be effective in GM@RES and GM@RES@VAN. The group showed a significant increase in HIF1A, MMP9, and PTGS2 indicators in GM@RES and GM@RES@VAN, the group significantly decreased. All indicators showed no statistical differences in the GM group. G. Compared with the control group, the key proteins of bone formation ALP, OPN, OCN, and COL I were found to be involved in GM@RES and GM@RES@VAN, the group significantly increased. All indicators showed no statistical differences in the GM group. ALP, OPN, COL I in GM@RES and GM@RES@VAN groups showed statistical differences (n = 3, *p < 0.05, **p < 0.01, ***p < 0.001).

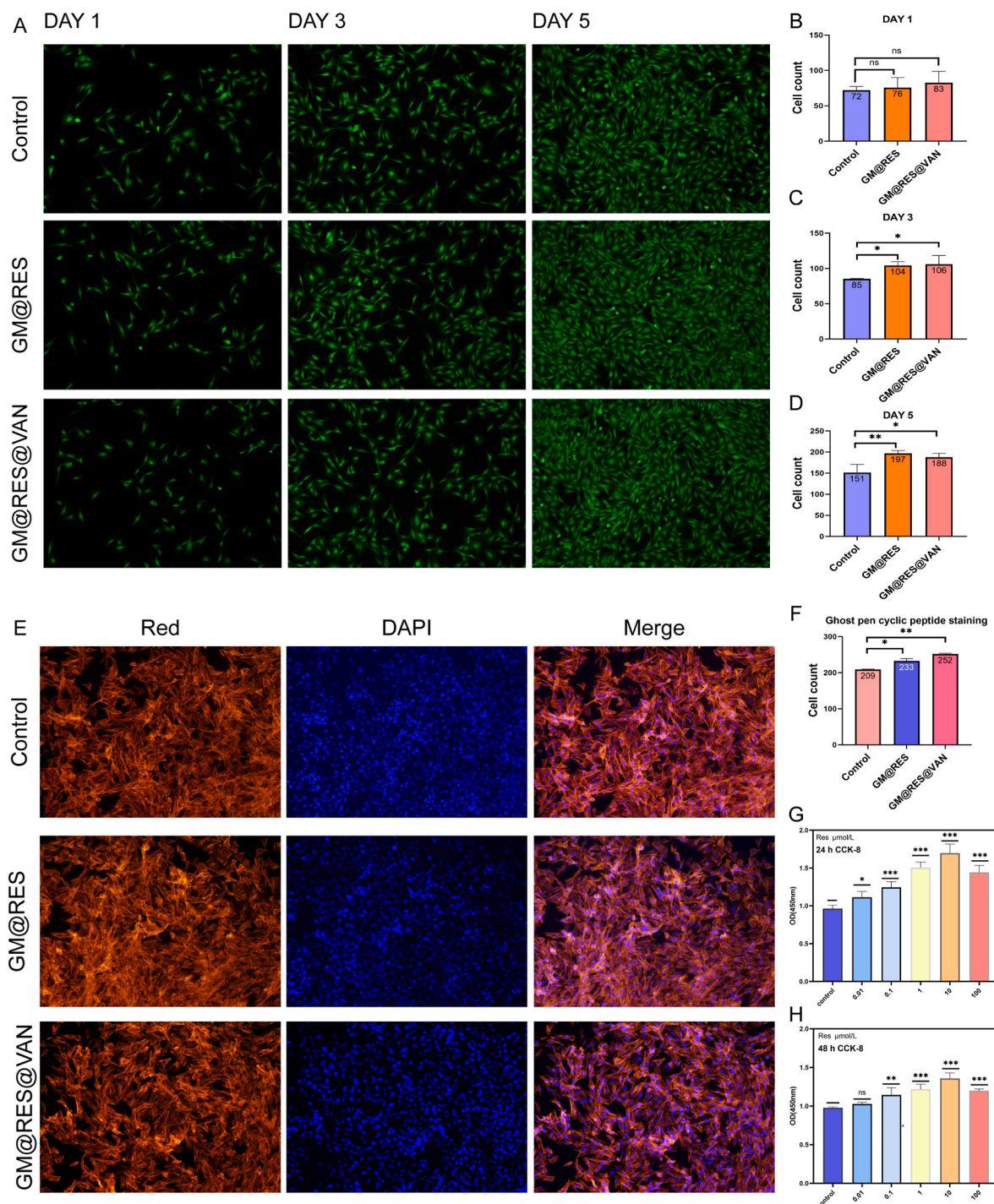


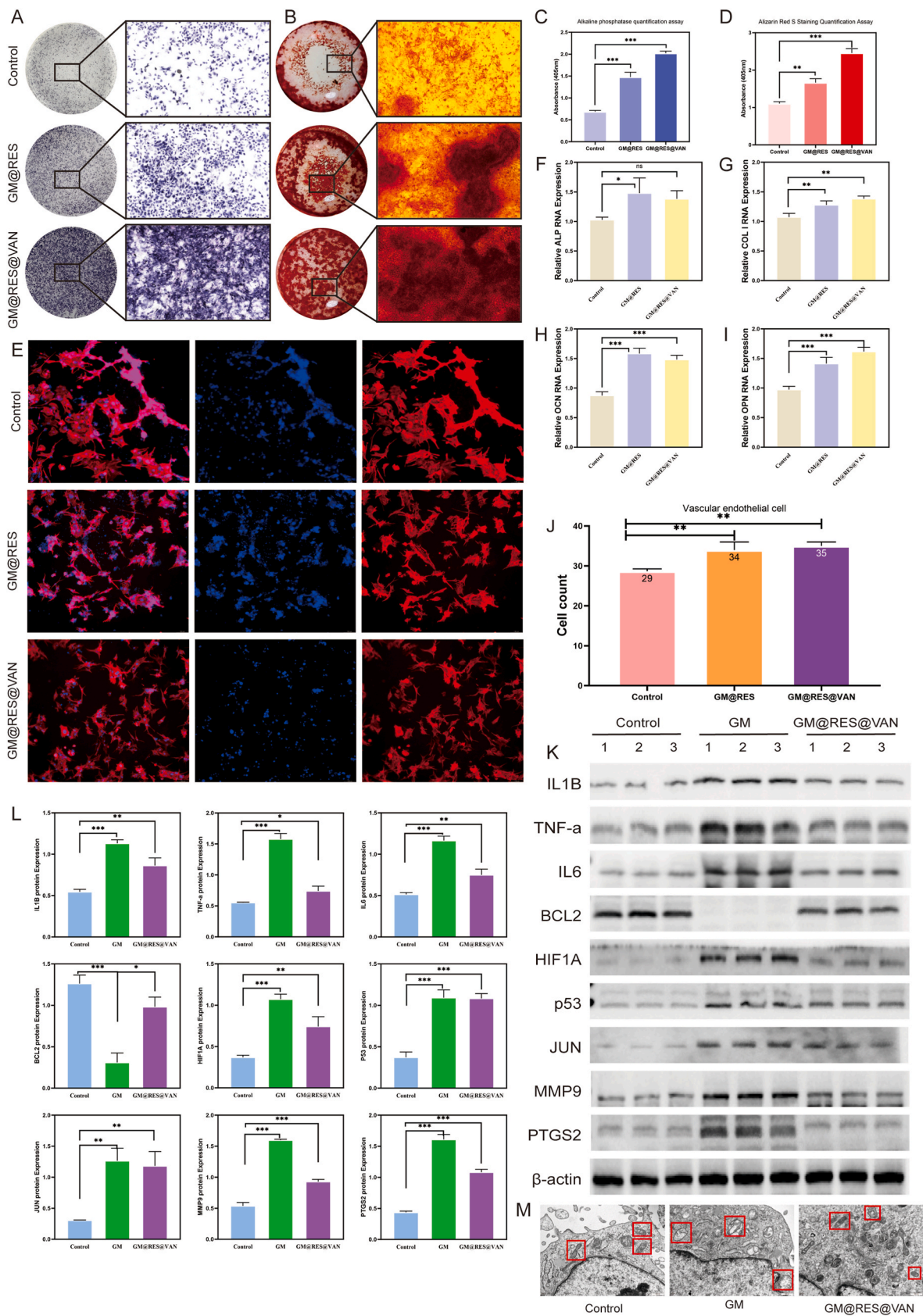
Fig. 8. Toxicity and biocompatibility testing of materials: A. Cell viability staining was used to evaluate the effects of different drug loaded microspheres on cell survival at 1, 3, and 5 days. B-D. As the culture time progressed, the number of cells gradually increased. The cell data of the GM@RES group and GM@RES@VAN group were higher than those of the control group at 3 and 5 days. E. The impact of different drug loaded microspheres on the cytoskeleton was evaluated using ghost pen peptide. F. The number of cells in the GM@RES group and GM@RES@VAN groups was higher than that in the control group under the evaluation of ghost pen peptide. G. CCK8 was used to determine the effect of drug loaded microspheres on cell proliferation within 24 and 48 h. Within 24 h, the cell proliferation efficiency is best at a dose of 10 μ mol/L ($n = 3$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

OPN, OCN, and COL I GM@RES group and GM@RES@VAN group showed significant expression, which is mainly related to the bone promoting ability of resveratrol (Fig. 9F–I).

The Western Blot results showed that in an infected environment, the expression of key inflammatory proteins such as IL1 β , IL-6, JUN, and TNF- α in the GM@RES@VAN group was significantly downregulated, while the anti apoptotic protein BCL2 was significantly expressed. The

hypoxia inducible factor HIF1A, extracellular matrix degrading MMP9, and inflammatory enzyme PTGS2 were all significantly reduced (Fig. 9K and L). This provides preliminary evidence that GM@RES@VAN plays a dual role in promoting osteogenesis and inhibiting inflammation at the cellular level by regulating signaling pathways such as apoptosis, autophagy, TNF, and HIF.

Under transmission electron microscopy, the mitochondria of the



(caption on next page)

Fig. 9. Results of osteogenic angiogenesis and mechanism verification: A-C. Alkaline phosphatase staining and quantitative analysis in different groups, compared with the control group, The alkaline phosphatase staining in GM@RES group and GM@RES@VAN group increased significantly with statistical significance ($n = 3$, $***p < 0.001$). B-D. Alizarin Red staining and quantitative analysis in different groups, compared with the control group, The increase in Alizarin Red staining in GM@RES and GM@RES@VAN group was significantly significant ($n = 3$, $**p < 0.01$, $***p < 0.001$). E-J. Immunofluorescence staining of endothelial cells in different groups was compared with the control group, The GM@RES and GM@RES@VAN group has more vascular formation. F. Compared with the control group, ALP, The expression of GM@RES group RNA has statistical significance ($n = 3$, $*p < 0.05$) There was no statistical significance in the GM@RES@VAN group ($n = 3$, NS). G. Compared with the control group, COL I, The RNA expression in the GM@RES and GM@RES@VAN group was statistically significant ($n = 3$, $***p < 0.01$), and H. Compared with the control group, OCN protein The RNA expression in the GM@RES and GM@RES@VAN group was statistically significant ($n = 3$, $***p < 0.01$). I. Compared with the control group, OPN protein, The RNA expression in the GM@RES and GM@RES@VAN group was statistically significant ($n = 3$, $***p < 0.001$). K. Band plots for the detection of 9 core proteins, L. Quantitative analysis and statistics for the detection of 9 core proteins, M. Control group, GM group, and Comparison of Mitochondria in GM@RES@VAN Group.

control group cells showed swelling, cristae breakage, and other forms of damage, while the mitochondrial damage in the blank microsphere group was further aggravated, indicating that the blank microspheres had no therapeutic effect. But the mitochondrial morphology of GM@RES@VAN group cells was restored and the cristae structure was clear, approaching a normal state, indicating that drug loaded microspheres may reduce the damage of infected environment to osteoblasts by protecting mitochondrial function (Fig. 9 M).

Western blot results from the HIF pathway inhibition experiment (Fig. 10A) showed that, compared with the blank group, the protein expression levels of HIF-1 α , inflammatory factors (IL1B, TNF- α , IL6), JUN, p53, MMP9, and PTGS2 were significantly increased in the MRSA-infected model group, while the anti-apoptotic protein BCL2 was significantly decreased. The blank microspheres (GM) had no significant ameliorative effect on any of the tested proteins. Compared with the model group, RES microspheres significantly down-regulated HIF-1 α and the aforementioned inflammation- and matrix-related proteins, and significantly up-regulated BCL2. Vancomycin microspheres showed a weak inhibitory effect on HIF-1 α and inflammatory factors, but significantly reduced JUN, p53, MMP9, and PTGS2, and moderately increased BCL2. The dual-drug microspheres (GM@VAN@RES) exhibited the strongest synergistic effect, with all measured indicators restored to levels close to those of the blank group. After the addition of the HIF-1 α inhibitor 2-ME, the protective effects of RES microspheres were significantly reversed: the inflammation- and matrix-related proteins increased again, and BCL2 decreased significantly. Although the dual-drug microspheres combined with the inhibitor still showed some improvement, the effect was much weaker than that of the combination without the inhibitor (Fig. 10B-J; NS, not significant; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). In summary, resveratrol exerts anti-inflammatory, anti-apoptotic, and matrix-regulating effects through a HIF-1 α -dependent pathway, whereas part of vancomycin's action is independent of this pathway. Their combination produces a synergistic effect, providing an important experimental basis for the treatment of MRSA infection-induced bone injury.

11. Discussion

The treatment of infected bone defects remains challenging because persistent bacterial infection triggers intense inflammatory responses and local hypoxia, severely interfering with bone regeneration. Conventional single-antibiotic therapy can control infection, but it has limited direct osteogenic potential and, with long-term use, raises concerns about drug resistance and impaired bone healing [33,34]. In this study, the GM@RES@VAN dual-drug loaded microsphere system integrates the potent antibacterial activity of vancomycin with the multiple bio-regulatory effects of resveratrol, demonstrating significant synergistic therapeutic efficacy in eradicating pathogens, remodeling the inflammatory microenvironment and promoting bone regeneration.

First, GM@RES@VAN microspheres exhibited excellent synergistic antibacterial performance, which is one of the bases for the synergy between resveratrol and vancomycin. In this study, GM@RES@VAN showed the strongest antibacterial activity and the highest biofilm degradation rate both in vitro and in vivo, significantly outperforming

vancomycin or resveratrol alone. This synergy may be achieved through multiple mechanisms. On the one hand, vancomycin exerts its classic bactericidal effect by specifically recognising and binding to the bacterial cell wall precursor D-Ala-D-Ala, rapidly eliminating planktonic bacteria. On the other hand, resveratrol, a plant polyphenol, is not merely a passive adjuvant but also possesses intrinsic antibacterial properties [35]. It can interfere with the expression of quorum-sensing system-related genes in *Staphylococcus aureus*, modulate the synthesis of surface proteins and capsular polysaccharides, thereby effectively inhibiting biofilm formation and disrupting pre-formed biofilms. By attacking bacteria from different sites, the two drugs act cooperatively, making GM@RES@VAN far more effective than a single antibacterial agent alone against methicillin-resistant *Staphylococcus aureus* and its tenacious biofilm, which is closely associated with infected bone defects [36].

Second, after infection is controlled, resveratrol plays a central regulatory role in repairing the damaged bone microenvironment. Infected bone defects often present high levels of inflammatory cytokines and severe hypoxia, both of which are major obstacles to osteogenesis. Through bioinformatics analysis and subsequent in vivo and in vitro experiments, this study confirmed that resveratrol exerts its anti-inflammatory and osteoblast-protective effects mainly by regulating the TNF/HIF signalling pathways. On the one hand, resveratrol significantly down-regulates key inflammatory factors such as IL-1 β , TNF- α and IL-6 by inhibiting the TNF signalling pathway [37–39]. It has been reported that resveratrol can suppress inflammation and promote osteogenic differentiation by modulating pathways such as NF- κ B and NRF2/HO-1. On the other hand, local infection often leads to aberrant high expression of HIF-1 α , which exacerbates tissue damage and inflammation [40,41]. The present study found that after resveratrol treatment, HIF-1 α levels in the infected environment were effectively reduced, consistent with a mechanism involving inhibition of the ROS/HIF pathway. More importantly, the HIF pathway inhibition experiment further validated this key mechanism: when the HIF pathway was blocked, the regulatory effects of resveratrol on inflammation- and apoptosis-related proteins were significantly reversed. This result not only reveals that the osteoprotective function of resveratrol is highly dependent on the HIF pathway, but also explains the molecular basis for its dual role in immunomodulation and osteogenesis in infected bone defects [42–44].

Finally, our data clearly demonstrate the spatiotemporal functional complementarity between resveratrol and vancomycin. Vancomycin acts as the “pioneer”, rapidly eliminating pathogens at the infection site, breaking the vicious cycle of inflammation sustained by persistent bacteria, and thus removing the obstacle to subsequent tissue repair [45]. After infection is effectively controlled, resveratrol takes over and exerts its anti-inflammatory and osteogenic effects: it improves the local inflammatory and hypoxic microenvironment by regulating the TNF/HIF pathways, down-regulates the expression of the extracellular matrix-degrading enzyme MMP9, up-regulates the anti-apoptotic protein BCL2, thereby enhancing the survival and function of osteoblasts; at the same time, it directly promotes the expression of osteogenic genes such as ALP, OPN, OCN and COL I, synergistically driving new bone formation. Notably, although the vancomycin-containing treatment

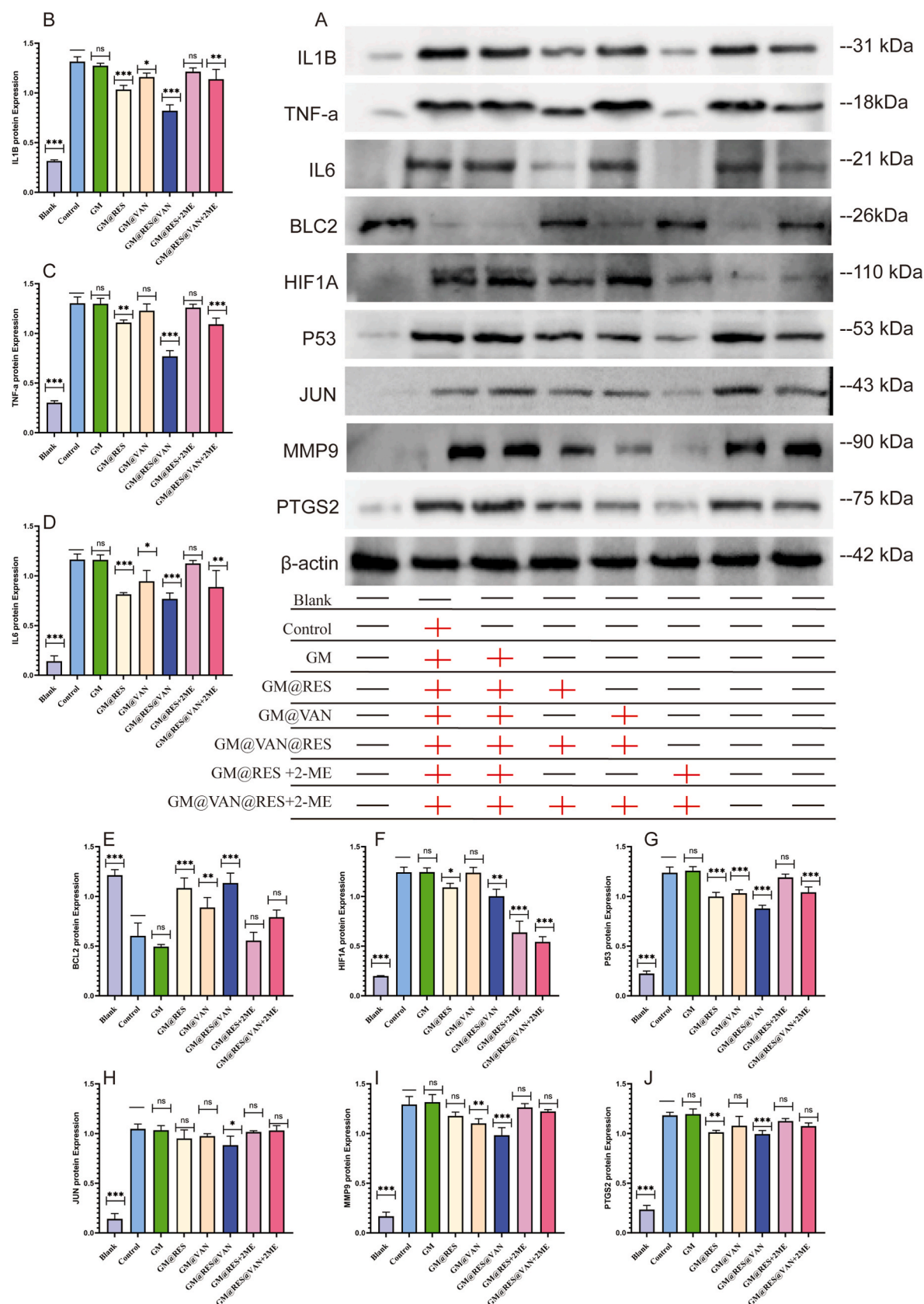


Fig. 10. A. Representative Western blots showing protein expression of HIF1A, IL1B, TNF- α , IL6, BCL2, JUN, p53, MMP9, PTGS2, and β -actin (loading control) in each group. (B–J) Densitometric quantification of the indicated proteins normalized to β -actin. Data are presented as mean $\pm$ SD ($n = 3$ independent experiments). NS: not significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the model group (Control) unless otherwise indicated by brackets. The results demonstrate that resveratrol exerts anti-inflammatory, anti-apoptotic, and matrix-regulating effects in a HIF-1 α -dependent manner, while vancomycin partially acts independently of HIF-1 α ; their combination produces synergistic effects.

group showed slightly better bone repair parameters than the RES-alone group, the differences were not statistically significant [17,46]. This suggests that vancomycin has a limited direct osteogenic effect; rather, its role in the combined therapy is mainly to create a favourable, low-inflammatory osteogenic microenvironment through potent antibacterial activity, thereby allowing the full osteogenic and anti-inflammatory potential of resveratrol to be realized (Fig. 11).

12. Limitations of the research and future solutions

In summary, this study successfully constructed a microfluidics-engineered GM@RES@VAN composite drug-loaded microsphere that combines the bioactive component of traditional Chinese medicine resveratrol with the antibiotic vancomycin, achieving a multifunctional synergy of “antibacterial anti-inflammatory osteopromotion.” Nevertheless, several limitations remain in this study.

First, the concentration of resveratrol used in our *in vitro* experiments (10 μM) is higher than the free plasma concentrations typically achieved under physiological conditions (usually submicromolar to low micromolar). Although 10 μM is a well-established effective concentration for promoting osteoblast differentiation in the literature (Mizutani et al., 1998; Bai et al., 2020), and although our microsphere-based sustained-release system is designed to achieve high local drug levels at the defect site—which can be orders of magnitude higher than systemic blood concentrations—we acknowledge that the direct translatability of these *in vitro* findings to the clinical setting remains to be established [47,48]. Future studies should measure the actual local drug concentration in bone tissue (e.g., by microdialysis) and use gradient dilutions of microsphere release supernatants to build a more precise *in vitro-in vivo* correlation.

Second, this study cannot fully distinguish whether the observed effects of resveratrol are specifically mediated by the identified targets or arise from its well-known broad antioxidant and anti-inflammatory activities (e.g., direct free radical scavenging, Nrf2 activation, NF- κB

inhibition). Although we supplemented an intervention experiment using a specific inhibitor of the HIF-1 α pathway (2-ME), which preliminarily demonstrated that part of resveratrol's effects depends on HIF signaling, we did not perform corresponding agonist/inhibitor interventions or gene knockdown/overexpression experiments for the remaining eight targets (e.g., IL1B, TNF, IL6, BCL2, JUN, TP53, MMP9, PTGS2). Therefore, the exact causal roles of these targets in resveratrol's anti-inflammatory, anti-apoptotic and bone-repair-promoting effects await further validation. We also cannot exclude the contribution of other off-target pathways (e.g., SIRT1, AMPK). Consequently, our conclusions should be interpreted with caution: the expression changes of these targets correlate with the therapeutic effects of resveratrol, and the necessity of the HIF-1 pathway has been preliminarily validated, but we do not claim that all effects are specifically mediated by the screened targets. Future studies employing siRNA, CRISPR-Cas9 knockout, or conditional knockout animal models are needed to systematically validate the causal role of each candidate target.

Third, although the animal model used in this study reasonably simulated infected bone defects, its pathological process still differs from the complex clinical infection microenvironment (e.g., polymicrobial infection, differences in immune status, mechanical loading). Future studies may consider further validating the safety and efficacy of this microsphere system in larger animal models (e.g., rabbits, dogs) or chronic infection models, and conducting causal validation for the above-mentioned unverified targets one by one to more comprehensively dissect the multi-target network of resveratrol.

Fourth, the animal study lacked two essential control groups: microspheres loaded with vancomycin alone (GM@VAN) and resveratrol alone (GM@RES), due to expected high mortality in GM@RES animals and institutional animal number restrictions. To partially address this deficiency, we performed complementary *in vitro* experiments that included both single-agent groups (GM@VAN and GM@RES) and demonstrated superior anti-inflammatory, anti-apoptotic, and HIF-1 α -dependent effects of the dual-drug microspheres (Fig. 10). However, in

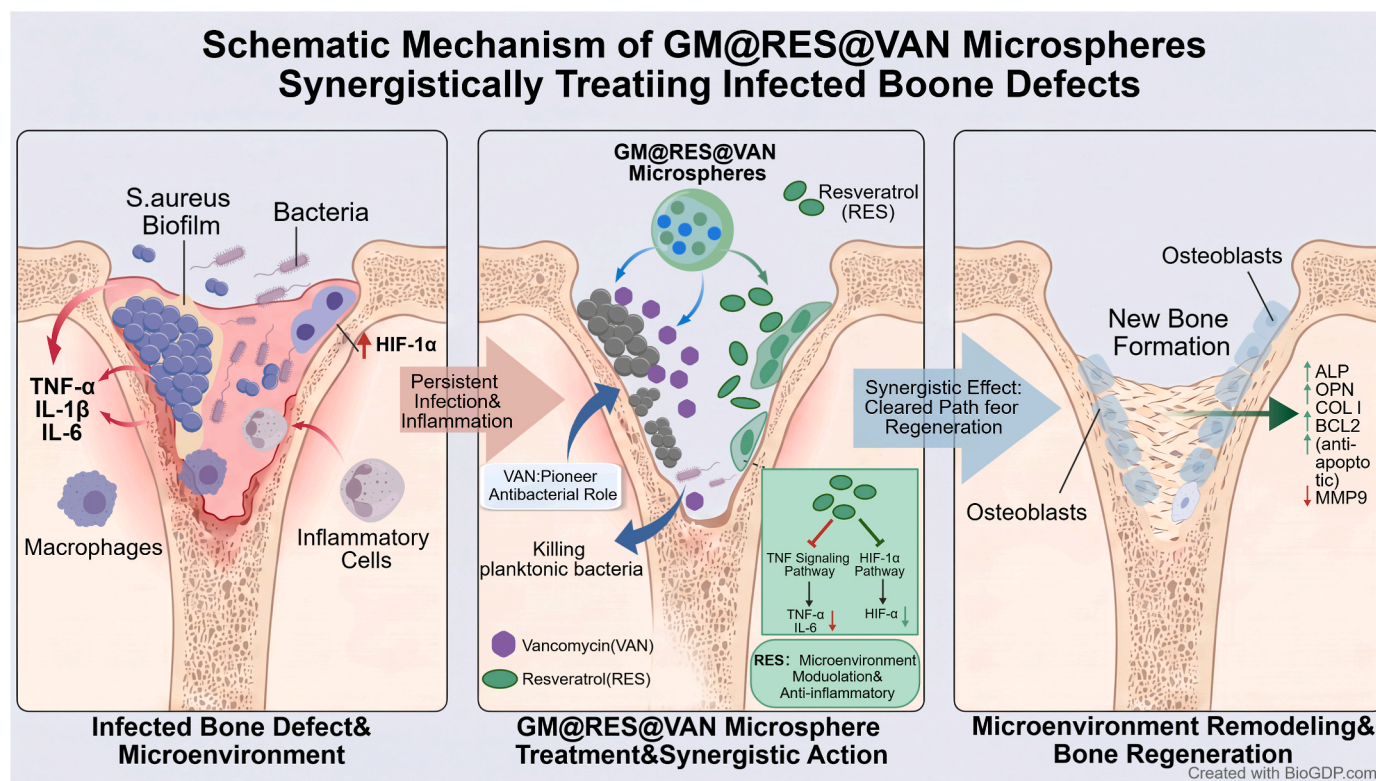


Fig. 11. Molecular mechanism of microfluidic construction of dual drug microspheres promoting infectious bone defects by reshaping the bone immune microenvironment through the TNF/HIF-1 signaling pathway.

vitro findings cannot fully recapitulate the complex in vivo environment. Owing to immediate graduation deadlines and expiration of animal facility access, we cannot re-run the long-term animal study with complete controls at this stage. This limitation is openly acknowledged, and a full factorial in vivo design (including both single-agent groups) is planned for future investigations.

13. Conclusion

In this study, we successfully constructed GM@RES@VAN microspheres using microfluidic technology, enabling spatiotemporal co-delivery of vancomycin and resveratrol. This system achieves early bacterial clearance by vancomycin and subsequent anti-inflammatory and osteogenic effects by resveratrol. Mechanistically, resveratrol simultaneously regulates the TNF and HIF-1 α signaling hubs, down-regulates IL-1 β , IL-6, and HIF-1 α , upregulates BCL2, preserves mitochondrial integrity, and inhibits MMP9 and PTGS2, thereby remodeling a bone-regenerative immune microenvironment. Although further validation using gene intervention and large-animal models is needed, this work provides a promising “antibacterial-anti-inflammatory-osteogenic” integrated strategy for infected bone defects and offers a novel paradigm for designing next-generation biomaterials combining natural products with conventional antibiotics.

Availability of data and material

The original data in this study can be found in the Supplementary Material. Additional data can be obtained upon request by contacting the corresponding author. The mechanism diagram of this study was drawn using the allstruion software drawing tool.

Ethics approval and consent to participate

The rats were all purchased from the Animal Experiment Center of Southwest Medical University, Certificate of Conformity: 44005800012441, and the experimental protocol was ethically approved by the Experimental Animal Welfare Ethics Committee of Southwest Medical University, approval No. 20231030-006 in its approval.

Consent for publication

Not applicable.

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ORCID iD authorship contribution statement

Chao Song: Formal analysis, Writing – original draft. **Yang Zhou:** Data curation, Software. **Yinjing Luo:** Investigation, Methodology. **Liquan Wang:** Formal analysis. **Tao Liu:** Resources. **Daqian Zhou:** Resources, Software. **Fei Liu:** Supervision. **Jingwen Chen:** Visualization. **Zhijiang Fu:** Funding acquisition, Methodology. **Feng Chen:** Conceptualization. **Guoyou Wang:** Writing – review & editing. **Zongchao Liu:** Conceptualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mtbio.2026.103326>.

Data availability

Data will be made available on request.

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