

Mitochondria-mimetic nanomedicine restores mitochondrial homeostasis to rescue traumatic and osteoporotic bone loss

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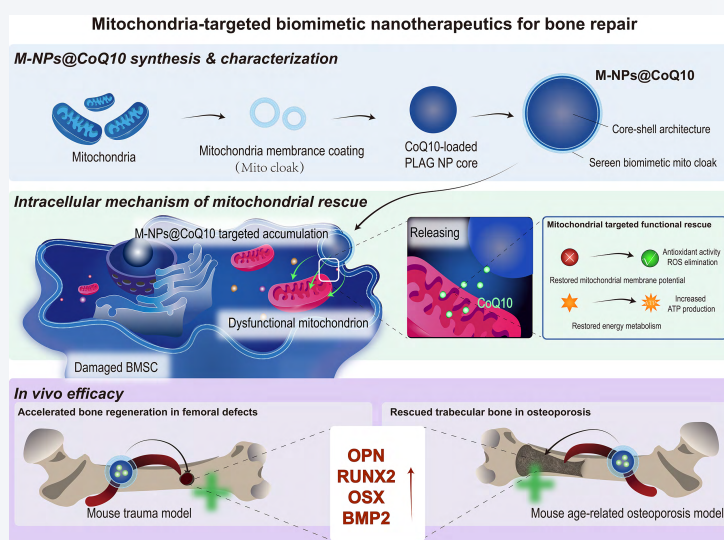
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ABSTRACT: Trauma-induced bone defects and age-related osteoporosis are prevalent osteogenic disorders in which impaired osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) remains a critical pathological challenge. Typically, BMSCs facilitate osteogenesis and increase bone mass by differentiating into osteoblasts; however, this differentiation is a high-energy process. In aging or trauma, BMSCs within the bone marrow exhibit substantial mitochondrial dysfunction, which severely limits their osteogenic potential and subsequent bone replenishment. Therefore, we developed a targeted biomimetic strategy to isolate mitochondria derived from BMSCs and formulated a membrane coating on poly(lactic-co-glycolic acid) nanoparticle cores, which were loaded with coenzyme Q10 (CoQ10) to facilitate mitochondrial repair. This engineered construct, M-NPs@CoQ10, demonstrates significantly enhanced cellular internalization and mitochondrial targeting. It exhibits superior efficacy in restoring mitochondrial function and promoting osteogenic differentiation both *in vitro* and *in vivo*. In murine models of trauma-mediated bone defects and age-induced osteoporosis, M-NPs@CoQ10 treatment achieved robust bone-recovery rates of 80% and 75%, respectively. This approach is a novel and highly effective therapeutic strategy for metabolic bone repair and related skeletal diseases.

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KEYWORDS: osteoporosis, mitochondria-mimetic nanoparticles, coenzyme Q10, osteogenic differentiation, drug delivery



1 Introduction

Skeletal integrity is frequently compromised by conditions, such as

trauma-induced bone defects and age-related osteoporosis, which collectively constitute considerable clinical challenges owing to their high morbidity and associated healthcare burdens. Traumatic injuries often result in critically sized bone defects that fail to spontaneously heal, necessitating secondary interventions to restore bone continuity and function [1]. Concurrently, osteoporosis, characterized by decreased bone mass and microarchitectural deterioration, predisposes patients to fragility fractures that substantially diminish their quality of life and increase mortality risk [2, 3]. Although bisphosphonates remain a cornerstone in osteoporosis management because they reduce fracture incidence,

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their therapeutic onset is slow and requires prolonged administration, which can lead to adverse events and limit their long-term use [4, 5]. Therefore, innovative therapeutic strategies are urgently needed to effectively modulate bone resorption and formation, accelerate the repair processes, and improve patient outcomes.

Mesenchymal stem cells (MSCs), a heterogeneous population of multipotent progenitors, have regenerative and immunomodulatory capabilities, rendering them promising candidates for bone tissue engineering and repair [6, 7]. Notably, osteogenic differentiation of bone marrow-derived MSCs (BMSCs) is a critical determinant of successful bone regeneration; however, this differentiation is energetically demanding and intimately dependent on optimal mitochondrial function [8]. Mitochondria serve as primary bioenergetic hubs, and their dysfunction, commonly observed in aging and post-injury microenvironments, impairs MSC survival and osteogenic potential, thereby severely limiting effective bone formation [9, 10]. This mitochondrial decline contributes to reduced adenosine triphosphate (ATP) production, elevated oxidative stress, and disruption of cellular homeostasis, all of which collectively hamper regenerative capacity. Therefore, therapeutic approaches capable of restoring or enhancing mitochondrial function may overcome these critical barriers in bone repair. CoQ10, a lipophilic quinone localized predominantly in the inner mitochondrial membrane, plays a pivotal role in electron transport during oxidative phosphorylation and functions as a potent antioxidant, mitigating lipid peroxidation and free radical damage [11, 12]. The age-related diminution of endogenous CoQ10 biosynthesis strictly correlates with mitochondrial dysfunction, exacerbating the oxidative stress and energy metabolism disturbances fundamentally implicated in osteoporosis and other degenerative pathologies [13, 14]. Despite its tremendous therapeutic potential, the clinical translation of CoQ10 supplementation is severely hindered by its poor aqueous solubility, limited oral bioavailability, and insufficient mitochondrial targeting, which collectively reduce its efficacy in restoring mitochondrial function and promoting cellular health [15, 16].

Advances in nanotechnology have introduced novel delivery platforms capable of improving CoQ10 stability, enhancing cellular uptake, and enabling targeted delivery to mitochondria, thereby potentially overcoming these pharmacokinetic limitations [17, 18]. Current research on mitochondria-targeted therapies for bone regeneration remains in its infancy, with substantial gaps in our understanding of the effective delivery of mitochondrial modulators and their mechanistic roles in osteogenesis. Although nanomaterials have been extensively explored for drug delivery and tissue engineering applications, few studies have systematically addressed the integration of biological mitochondrial membranes as biomimetic coatings to enhance nanoparticle (NP) mitochondrial tropism and biocompatibility [8, 19]. The use of mitochondria-membrane-coated NPs represents a highly innovative biomimetic strategy. It combines the natural targeting capabilities and immune evasion properties of mitochondria with the controlled release and protective characteristics of biodegradable polymers, such as poly(lactic-co-glycolic acid) (PLGA). This approach could facilitate the highly efficient intracellular delivery of therapeutics, such as CoQ10, directly to mitochondrial compartments, thereby profoundly augmenting their therapeutic efficacy in bone repair and remodeling.

In this study, we developed a mitochondrial membrane-coated

PLGA NP system encapsulating CoQ10 (M-NPs@CoQ10) to enhance mitochondrial targeting and restore mitochondrial function in osteogenic cells. The NPs were synthesized using a single-emulsion technique with subsequent coating using isolated mitochondrial membranes derived from BMSCs, conferring highly specific biomimetic properties. This innovative delivery platform aims to leverage the dual benefits of mitochondrial membrane-mediated targeting and sustained-release kinetics of PLGA to maximize CoQ10 bioavailability within the mitochondria. Our objectives included evaluating the physicochemical characteristics, cellular uptake efficiency, and biocompatibility of M-NPs@CoQ10, and comprehensively assessing its potential to modulate mitochondrial function and promote osteogenic differentiation *in vitro*. Ultimately, this study seeks to establish a robust foundational framework for biomimetic, mitochondria-targeted nanotherapeutics to address critical challenges in bone defect repair and osteoporosis treatment.

2 Experimental

2.1 Materials

PLGA (M_w 20,000, 50:50) was purchased from Xi'an Ruixi Biological Technology (China). CoQ10, fluorescent dyes (fluorescein isothiocyanate (FITC) and Cy5.5), and a tetramethylrhodamine methyl ester (TMRM) probe were obtained from MedChemExpress (MCE, USA). MitoSOX superoxide indicator (M36005) and plasma membrane dyes (C10046) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Phosphate-buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), Penicillin-Streptomycin, and trypsin were purchased from Biosharp (China). Mouse BMSCs, along with osteogenic differentiation medium and osteogenic induction factors, were acquired from Cyagen Biosciences (China). The Coomassie Blue staining solution, cell counting kit-8 (CCK-8), Cell Mitochondria Isolation Kit, Mammalian Genomic DNA Extraction Kit, ATP Assay Kit, alkaline phosphatase (ALP) Assay Kit, Masson's trichrome staining kit, hematoxylin and eosin staining (H&E) kit, and EDTA decalcifying solution were purchased from Beyotime (China). Hydrogen peroxide solution was purchased from Aladdin (China). The reverse transcriptase was obtained from Yeasen Biotechnology (Shanghai, China). The antibodies against runt-related transcription factor 2 (RUNX2, cat. no. SD208-0), osterix (OSX, cat. no. PSH07-29), and bone morphogenetic protein 2 (BMP2, cat. no. ER80602) were obtained from HUABIO (China). The antibodies against succinate dehydrogenase A (SDHA, cat. no. 14865-1-AP), osteopontin (OPN, cat. no. 83341-1-RR), histone H3 (H3, cat. no. 17168-1-AP), translocase of the outer mitochondrial membrane 20 (TOM20, cat. no. 11802-1-AP), cytochrome *c* oxidase subunit IV (COXIV, cat. no. 11242-1-AP), calnexin (CANX, cat. no. 10427-2-AP), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α , cat. no. 66369-1-Ig), mammalian target of rapamycin (mTOR, cat. no. 66888-1-Ig), p-mTOR (cat. no. 67778-1-Ig), AMP-activated protein kinase alpha subunit (AMPK α , cat. no. 10929-2-AP), p-AMPK α (cat. no. 80209-6-RR), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, cat. no. 60004-1-Ig) were purchased from Proteintech (China). Enzyme-linked immunosorbent assay (ELISA) kits for aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK), and blood urea nitrogen (BUN) were obtained from eBioscience (USA).

2.2 Animals

For subsequent trauma-induced bone defect and age-related osteoporosis modeling, adult (8-week-old) and aged (18-month-old) male C57BL/6 mice were used. Male C57BL/6 mice were purchased from Hangzhou Medical College. Age-matched mice were randomly assigned to each experimental group and housed in a specific pathogen-free facility under controlled conditions (temperature < 25 °C, humidity ~ 50%, 12 h light/dark cycle), with free access to food and water. Prior to sacrifice, venous blood was collected from the ocular veins of the mice and allowed to clot for 30 min at 25 °C. Serum was separated using centrifugation at 3000 rpm for 10 min and stored for subsequent assessment of cardiac, renal, and hepatic functions.

2.2.1 Animals image

For biodistribution analysis, mice were intravenously injected with DiR-labeled nanoparticles (200 μ L, 1 mg/mL). At predetermined time points (24 and 48 h), mice were euthanized by cervical dislocation. Major organs including the heart, liver, spleen, lung, kidney, and bilateral femurs were harvested and immediately imaged using an IVIS Spectrum *in vivo* imaging system (PerkinElmer, USA) with excitation/emission filters set at 745/800 nm. Fluorescence signals were quantified as radiant efficiency ((photons/s/cm²/sr)/(μ W/cm²)) using Living Image software (PerkinElmer, USA).

2.3 Synthesis of blank PLGA NPs and fluorescent labeling

Blank PLGA NPs were synthesized using the standard oil-in-water (O/W) single-emulsion solvent evaporation method. PLGA (60 mg) was dissolved in 2 mL of dichloromethane (DCM) to form the organic phase. This solution was added directly to 20 mL of 2% (w/v) poly(vinyl alcohol) (PVA) aqueous solution. The mixture was immediately emulsified using a probe sonicator (30 W) in an ice-water bath for 5 min (3 s on, 2 s off cycle). Following emulsification, the resulting suspension was continuously stirred in an open container at 25 °C for 3–4 h until the liquid became completely translucent, indicating full evaporation of the organic solvent. To remove large polymer aggregates, the suspension was first subjected to low-speed centrifugation at 1000g for 5 min. The supernatant was carefully collected and centrifuged at a high speed of 12,000g for 15 min to pellet the NPs. Finally, blank PLGA NPs were washed with deionized (DI) water, recovered by high-speed centrifugation, and resuspended in ultrapure water for subsequent experiments.

To prepare fluorescently labeled NPs, the synthesis procedure was identical to that of the bare NPs, except that 0.5 mg of a hydrophobic fluorescent dye (Cy5.5 or FITC) was co-dissolved with PLGA in the DCM organic phase prior to emulsification. All subsequent procedures were performed in the dark to prevent photobleaching.

2.4 Synthesis of CoQ10-loaded PLGA NPs (NPs@CoQ10)

NPs@CoQ10 were prepared using an O/W single-emulsion solvent evaporation method. PLGA (60 mg) and 6 mg CoQ10 were dissolved in 2 mL of DCM to form the organic phase. This organic solution was then added dropwise into 20 mL of a 2% (w/v) PVA aqueous solution. The mixture was immediately emulsified using a probe sonicator (300 W) in an ice-water bath for 5 min, with a 3 s on, 2 s off cycle. Subsequently, the resulting emulsion was

continuously stirred in an open container at 25 °C for 3–4 h to allow complete evaporation of the organic solvent. To remove unencapsulated CoQ10 crystals and large polymer aggregates, the suspension was centrifuged at a low speed of 1000g for 5 min. The supernatant was carefully collected and further centrifuged at 12,000g for 15 min to pellet the NPs@CoQ10. The obtained NPs were washed once with DI water, collected via centrifugation, and resuspended in ultrapure water. Empty PLGA NPs were prepared following an identical procedure, but without the addition of CoQ10.

2.5 Mitochondrial extraction

For mitochondria extraction, we initiated the process using approximately 20 million harvested mouse BMSCs. The cells were washed with ice-cold PBS and collected via centrifugation. To release the intracellular components, the BMSC pellet was resuspended in 1 mL of Mitochondrial Isolation Reagent A, supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) to prevent protein degradation, and incubated on ice for 10 to 15 min to induce hypotonic swelling. Subsequently, the cells were mechanically disrupted by applying 30 strokes to a Dounce glass homogenizer. We then used a differential centrifugation strategy to separate the mitochondria from the crude homogenate. Specifically, an initial low-speed centrifugation at 1200 $\times$ g for 10 min at 4 °C was performed to precipitate and remove intact cells and nuclei. The mitochondria-rich supernatant was carefully transferred to a new tube and subjected to a high-speed centrifugation at 12,000 $\times$ g for 10 min at 4 °C. Ultimately, the resulting pellet, which comprised the isolated crude mitochondria, was collected and gently resuspended in 500 μ L of Mitochondrial Isolation Reagent A for subsequent membrane derivation.

2.6 Preparation of biomimetic mitochondrial membrane-coated NPs (M-NPs@CoQ10)

M-NPs@CoQ10 were fabricated using sonication and mechanical co-extrusion. Based on the bicinchoninic acid (BCA) quantification results, the extracted mitochondrial membrane and pre-synthesized NPs@CoQ10 were mixed in 1 $\times$ PBS at a membrane protein-to-PLGA polymer weight ratio of 1:1. The mixture was initially sonicated in a 4 °C water bath for 5 min to facilitate the preliminary fusion of the biological membranes with the polymeric cores. Subsequently, the mixture was serially extruded through 400 and 200 nm polycarbonate porous membranes using a mini-extruder with 20 passes through each pore size. The mechanical shear force generated during the extrusion process promoted complete and uniform coating of the mitochondrial membranes onto the surface of NPs@CoQ10. Finally, the extruded dispersion was centrifuged at 12,000 $\times$ g for 10 min to remove unbound, free mitochondrial membranes. The purified M-NPs@CoQ10 pellets were resuspended in 1 $\times$ PBS and stored at 4 °C for subsequent *in vitro* and *in vivo* experiments.

2.7 Material characterization

The hydrodynamic diameter (particle size), polydispersity index, and zeta potential of the bare NPs, isolated mitochondrial membranes, and biomimetic NPs (M-NPs) were determined using dynamic light scattering (DLS). To evaluate the colloidal stability of the formulation, bare NPs and M-NPs were dispersed in 1 $\times$ PBS (pH 7.4) or 1 $\times$ PBS supplemented with 10% (v/v) fetal bovine serum (FBS). The suspensions were incubated at 25 °C for 7 days.

During this period, an aliquot from each sample was collected daily, and dynamic changes in particle size were monitored using DLS. The successful isolation of mitochondrial membranes and biomimetic core-shell structure of the assembled M-NPs were verified using transmission electron microscopy (TEM). Diluted samples were dropped onto carbon-coated copper grids and allowed to adhere. The grids were then negatively stained with uranyl acetate solution and air-dried prior to visualization using TEM. Furthermore, to confirm the successful translocation and retention of mitochondrial membrane proteins onto the polymeric cores, 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted. Isolated mitochondrial membranes (containing 10 μg of total protein) and M-NPs (containing an equivalent 10 μg of membrane protein) were mixed with loading buffer, denatured by boiling, and separated on a polyacrylamide gel. Following electrophoresis, specific protein profiles were visualized using Coomassie Brilliant Blue staining. To obtain the drug release profile, 4 mg of material was dispersed in 4 mL deionized water and placed in a dialysis apparatus. PBS (1 mM, pH 7.4) was prepared as the dialysis buffer. At specific time points (1, 2, 4, 8, 24, 48, and 72 h), 100 μL of the material dispersion was withdrawn from the dialysis apparatus, and the peak area was measured by sample injection. The cumulative drug release profile was obtained by calculating the ratio of the drug peak area at each time point to the initial drug content.

2.8 *In vitro* cytotoxicity assay

The *in vitro* cytotoxicity of the formulated NPs was evaluated using a standard CCK-8 assay. BMSCs in the logarithmic growth phase were harvested and seeded into 96-well plates at a density of 5×10^3 cells per well in 100 μL of complete culture medium. The plates were then incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h to allow for complete cell attachment. The original culture medium was then carefully removed. The cells were divided into three groups and treated with 100 μL of fresh medium containing either bare NPs (200 $\mu\text{g}/\text{mL}$) or M-NPs (at an equivalent PLGA core concentration of 200 $\mu\text{g}/\text{mL}$). Cells cultured in a fresh medium without any NP treatment served as negative controls. After 48 h of co-incubation, the NPs-containing medium in each well was discarded and cells were gently washed once with PBS to eliminate any background interference from residual nanomaterials. Next, 100 μL of fresh culture medium containing 10% (v/v) CCK-8 reagent was added to each well. Following an additional incubation for 1.5 h in the dark at 37 °C, the absorbance (OD value) of each well was measured at a wavelength of 450 nm using a microplate reader. The cell viability was calculated relative to that of the untreated control group.

2.9 *In vitro* mitochondrial damage model and treatment

To establish an *in vitro* model of oxidative stress-induced mitochondrial dysfunction, BMSCs were seeded into six-well plates at a density of 1.5×10^5 cells per well and incubated overnight to allow complete cell attachment. Subsequently, the cells were exposed to 50 μM hydrogen peroxide (H_2O_2) for 12 h to induce mitochondrial damage. Following oxidative stimulation, the H_2O_2 -containing medium was carefully removed and cells were divided into three experimental groups for subsequent assays: (1) the control group, which received fresh standard culture medium; (2) NPs@CoQ10 group, treated with fresh medium containing bare NPs@CoQ10; and (3) M-NPs@CoQ10 group, treated with fresh medium containing biomimetic M-NPs@CoQ10. Treatments

across all groups were normalized to an equivalent PLGA core concentration of 200 $\mu\text{g}/\text{mL}$.

2.10 Evaluation of mitochondrial function

To assess the mitochondrial membrane potential ($\Delta\Psi\text{m}$), BMSCs subjected to the aforementioned treatments (Control, NPs@CoQ10, and M-NPs@CoQ10) were evaluated using TMRM staining. This was performed at two levels: whole cells and isolated organelles. For whole-cell analysis, the treated BMSCs were directly incubated with the TMRM fluorescent probe in the dark at 37 °C for 10 min. Alternatively, mitochondria were extracted from treated cells using the previously described isolation protocol and subsequently stained with TMRM. The fluorescence intensity, which is directly proportional to the potential, was recorded to evaluate the mitochondrial state. To assess mitochondrial superoxide levels, BMSCs treated as described above were incubated with MitoSOX superoxide indicator at 37 °C in the dark for 10 min, followed by detection and recording of fluorescence intensity. Furthermore, to comprehensively determine mitochondrial metabolic function and energy production capacity, intracellular ATP levels of the equivalently treated BMSCs were quantified. The treated cells were lysed, ATP content was measured using a commercial ATP assay kit according to the manufacturer's instructions, and luminescence signals were detected using a microplate reader.

2.11 Evaluation of mitochondrial DNA (mtDNA) content

To evaluate the cellular mtDNA content, total intracellular DNA (comprising both nuclear and mitochondrial DNA) was isolated from the variably treated BMSCs using a commercial Mammalian Genomic DNA Extraction Kit, following the manufacturer's protocols. The relative mtDNA copy number was determined using quantitative PCR (qPCR). The mtDNA content was calculated by determining the ratio of the mitochondria-encoded 16S rRNA gene to the nuclear-encoded housekeeping gene, β -actin. The relative fold change in mtDNA copy number was analyzed using the $2^{-\Delta\Delta\text{Ct}}$ method, with the untreated cells serving as the baseline control.

2.12 BMSCs osteogenic differentiation

To induce osteogenic differentiation, mouse BMSCs were cultured in an osteogenic induction medium. BMSCs were seeded at an appropriate density on gelatin-coated culture plates to enhance cell attachment. When the cells reached approximately 70%–80% confluence, the growth medium was replaced with an osteogenic induction medium. This medium consisted of high-glucose DMEM supplemented with 50 $\mu\text{g}/\text{mL}$ ascorbic acid, 10 mM β -glycerophosphate, and 100 nM dexamethasone. The induction medium was refreshed every 2–3 days using the half-medium change method. After a designated induction period (7 days), ALP activity, an early marker of osteogenesis, was assessed. Cells were fixed and stained using a commercial ALP staining kit according to the manufacturer's instructions. The stained cells were then imaged under a light microscope. For quantitative analysis, the stained samples were dissolved in a 0.5 M HCl solution containing 5% SDS, and the absorbance was measured at 405 nm.

2.13 Establishment of osteogenesis disorder models and treatments

2.13.1 Femoral defect model

To establish the bone defect model, 8-week-old male C57BL/6 mice

were anesthetized using an intraperitoneal injection of 1% pentobarbital sodium at a dose of 50 mg/kg. After hair removal and routine disinfection, a longitudinal incision was made to bluntly separate the muscle and expose the femoral shaft. A perforated full-thickness defect was drilled into the middle of the right femur using a 1 mm disposable biopsy punch with a plunger (Integra Miltex). The surgical area was flushed with sterile saline solution to remove bone chips and the incision was closed in layers with sterile silk sutures. For postoperative analgesia, 50 μ L of 0.04 mg/mL buprenorphine hydrochloride was subcutaneously injected every 12 h for 2 days. Post-surgery, the mice were randomly assigned to four groups: the Vehicle (age-matched healthy mice without surgery), Control (surgery followed by injection of 200 μ L of PBS), NPs@CoQ10 (surgery followed by injection of 200 μ L of NPs@CoQ10), and M-NPs@CoQ10 group (surgery followed by injection of 200 μ L of M-NPs@CoQ10). For the treatment groups, formulations were administered via tail vein injection once a week starting from the day of surgery, with an injection volume of 200 μ L per mouse (10 mg/mL NPs). At the designated experimental endpoint of 8 weeks post-surgery, the mice were euthanized using CO₂ asphyxiation. The femora were fully dissected, harvested, and fixed in 4% paraformaldehyde (PFA) for 24 h for subsequent micro-computed tomography (micro-CT) and histological analyses.

2.13.2 Age-related osteoporosis model

To evaluate the systemic osteoprotective effects of biomimetic NPs, a natural age-related osteoporosis model was used. The experimental groups were designed as follows: the Vehicle group consisted of 8-week-old healthy male mice serving as the young baseline control; Control group comprised 18-month-old naturally aging mice treated with 200 μ L of PBS; and NPs@CoQ10 and M-NPs@CoQ10 groups consisted of 18-month-old aging mice receiving the respective NP treatments. The therapeutic intervention protocol was identical to that of the bone defect model: mice in the treatment groups received weekly tail vein injections of the formulations, with an injection volume of 200 μ L per mouse (10 mg/mL NPs), for a continuous duration of 8 weeks. At the 8-week experimental endpoint, all the mice were euthanized using CO₂ asphyxiation. The bilateral femora were collected and fixed in 4% PFA for 24 h to assess the extent of bone microarchitectural degeneration and therapeutic regeneration.

2.14 Western blotting

Western blotting was performed as previously described with some modifications. Total protein extraction was conducted on cells or tissues using radioimmunoprecipitation assay (RIPA) lysis buffer (Fude Biological Technology, cat. no. FD009; China) supplemented with protease and phosphatase inhibitors. The protein concentration was determined using a BCA assay (Beyotime, cat. no. P0010S). Equal amounts of protein were separated using SDS-PAGE and subsequently transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking with 5% non-fat milk at 25 °C for 2 h and washing with TBST buffer (Fude Biological Technology, cat. no. FD9058), the membrane was incubated with primary antibodies (RUNX2, 1:1500; BMP2, 1:1500; OSX, 1:1500; OPN, 1:1500; TOM20, 1:1500; COXIV, 1:1500; H3, 1:1500; CANX, 1:1500; TUBA1B, 1:1500; PGC-1 α , 1:1500; AMPK, 1:1500; p-AMPK, 1:1000; mTOR, 1:1500; p-mTOR, 1:1000; GAPDH, 1:2000) overnight at 4 °C, followed by incubation with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (sheep anti-mouse IgG, 1:5000, SA00001-1, Proteintech; sheep anti-

rabbit IgG, 1:5000, SA00001-2, Proteintech) for 1.5 h at 25 °C. Protein bands were visualized using an enhanced chemiluminescence detection system (Fude Biological Technology, cat. no. FD8020).

2.15 Immunofluorescence

The sections were deparaffinized and hydrated. After antigen retrieval using pepsin solution (G0142-100ML, Servicebio) at 4 °C for 30 min, the sections were incubated with 3% H₂O₂ and 5% bovine serum albumin (BSA) at 25 °C for 20 min and with the indicated antibodies at 4 °C overnight. Subsequently, secondary Alexa Fluor 488 goat anti-rabbit IgG (1:500) was added to the sections. The slides were mounted with antifade mounting medium containing DAPI (HKI0007-2, HaoKeBio). Representative images were captured using a whole-slide scanner (KF-PRO-120, KFBIO, China) and the relative fluorescence intensity was quantified using ImageJ v1.50.

2.16 Histology

The mouse femurs were fixed in formalin, embedded in paraffin, and sectioned. For histological analysis, the tissue sections were stained with H&E (Beyotime, cat. no. C0105S) and Masson's trichrome (Beyotime, cat. no. C0189S) staining. The paraffin-embedded sections were deparaffinized in xylene and rehydrated in a graded ethanol series. For H&E staining, the sections were stained with hematoxylin, differentiated, blued, and counterstained with eosin. For Masson's trichrome staining, sections were sequentially stained with Weigert's iron hematoxylin, Biebrich scarlet-acid fuchsin, and aniline blue according to standard protocols to distinguish collagen fibers (blue) from muscle fibers (red). After dehydration and clearing, all sections were mounted with neutral balsam and representative images were acquired using a whole-slide scanner (KF-PRO-120, KFBIO).

2.17 Micro-CT

Micro-CT analysis was performed to evaluate bone microarchitecture. The harvested bone specimens were fixed in 4% PFA for 48 h, washed, and stored in 70% ethanol. The samples were then scanned using a high-resolution micro-CT scanner (Skyscan 1072, Bruker) in 17 mm scanning tubes with an 11 mm³ volume at 180 mA, 50,000 V, and an acquisition time of 8 min. After scanning, three-dimensional reconstruction was performed using the manufacturer's software (NRecon, Bruker). Quantitative morphometric parameters, bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular separation (Tb.Sp), from the region of interest (ROI) were analyzed using the CTAn analysis software (Bruker). Representative micro-CT images were generated using micro-CT analysis software (CTVox, Bruker).

2.18 Quantitative PCR analysis

Total mRNA was extracted using TRIzol and the SteadyPure Universal RNA Extraction Kit following the manufacturer's protocols. Subsequent complementary DNA (cDNA) synthesis was performed using the Evo MMLV RT Premix. Real-time qPCR was performed using SYBR Green Master Mix (Yeason, China) in a QuantStudio 5 Flex Real-Time PCR System (Thermo Fisher Scientific), following the manufacturer's guidelines. The *C_t* values of each gene were normalized using the *C_t* value of β -actin for real-time qPCR. The primer sequences are listed below:

β-Actin: Forward 5'-GGCTGTATTCCCCTCCATCG-3'; Reverse 5'-CCAGTTGGTAACAATGCCATGT-3';

Runx2: Forward 5'-GACTGTGGTTACCGTCATGGC-3'; Reverse 5'-ACTTGGTTTTTCATAACAGCGGA-3';

Opn: Forward 5'-CCTGGCTGAATTCTGAGGGAC-3'; Reverse 5'-TTCTGAGATGGGTCAGGCAC-3';

Bmp2: Forward 5'-GGGACCCGCTGTCTTCTAGT-3'; Reverse 5'-TCAACTCAAATTCGCTGAGGAC-3';

Osx: Forward 5'-GATGGCGTCTCTCTGCTTG-3'; Reverse 5'-GGGCTGAAAGGTCAGCGTAT-3';

Sdha: Forward 5'-ACTAGGGCTGGTTTGCTTG-3'; Reverse 5'-AATTCGGGTAGACGTGTGG-3'.

2.19 Statistical analysis

All experiments were repeated independently at least three times and only data from representative experiments are presented. The results are presented as the mean $\pm$ standard deviation (SD), and group sizes are indicated in figure legends. All datasets were analyzed for normality and variance. Statistical analysis was performed using one-way analysis of variance with Tukey's post-hoc test for intergroup comparisons using GraphPad Prism software version 9.5 with a P value < 0.05 considered to be statistically significant.

3 Results and discussion

3.1 Synthesis, characterization, and mitochondrial internalization of M-NPs

To obtain mitochondrial membranes, namely Mito cloak, we isolated mitochondria from a bulk culture of BMSCs for subsequent encapsulation in PLGA NPs. TEM confirmed the successful isolation of structurally intact mitochondria, which exhibited a typical elongated oval shape with well-preserved double-membrane structures and clearly visible cristae invaginations at both low (5000 $\times$) and high (50,000 $\times$) magnification (Fig. 1(a)). Subsequently, the mitochondrial membranes were coated onto the pre-synthesized NPs via a combination of ultrasonication and mechanical co-extrusion. TEM imaging revealed that the isolated mitochondrial membrane (Mito cloak) appeared as a hollow, irregularly shaped spherical structure. In contrast, M-NPs displayed a distinct core-shell architecture characterized by electron-dense spherical cores surrounded by a relatively lighter outer layer. This morphology is consistent with that of the NP cores coated with the mitochondrial membrane. The uniform spherical shape and presence of a surface coating on the M-NPs, as opposed to the irregular membrane vesicles, provide direct visual evidence for the successful fabrication of mitochondria-mimetic NPs (Fig. 1(b)). After the successful synthesis of M-NPs, their physical properties were characterized. The hydrodynamic diameter and size distribution of the Mito cloak, NPs, and M-NPs were characterized using DLS. The NPs exhibited a narrow size distribution with a peak intensity at approximately 150 nm. The M-NPs displayed a slightly larger hydrodynamic diameter with a peak centered at approximately 200 nm, supporting the successful coating of the mitochondrial membrane onto the NP surface. In contrast, the mitochondria showed a distinct population size with a peak intensity at approximately 2000 nm, which was significantly larger than that of both NPs and M-NPs (Fig. 1(c)). The shift in size distribution from NPs to M-NPs indicates the formation of a core-

shell structure, while the absence of a Mito-sized population in the M-NP sample suggests effective purification and removal of free mitochondria following the coating process. DLS indicated that the average diameter of M-NPs (210.63 ± 4.34 nm) was slightly larger than that of uncoated NPs ($P < 0.05$) (161.33 ± 6.52 nm), and the surface charge of M-NPs (-28.80 ± 1.74 mV) was equivalent to that of Mito cloak (-31.03 ± 2.94 mV) but less negative than that of uncoated NPs ($P < 0.05$) (-25.5 ± 1.08 mV, Fig. 1(d)). Both TEM and DLS data suggested the successful surface coating of the Mito cloak on the NPs.

To systematically characterize the biological properties of M-NPs, we performed a series of evaluations. To confirm that membrane proteins were retained during the membrane-coating process, we evaluated M-NPs using Coomassie brilliant blue staining (Fig. 1(e)). The protein profile of the M-NPs closely resembled that of the Mito cloak, implying the preservation of mitochondrial components. Additionally, western blot showed mitochondrial markers (TOM20 and COXIV) were significantly enriched in both Mito cloak and M-NPs, confirming successful protein preservation during mechanical co-extrusion. In contrast, markers for the nucleus (H3), cytoplasm (TUBA1B), and endoplasmic reticulum (CANX) were absent in M-NPs (Fig. S1 in the Electronic Supplementary Material (ESM)), demonstrating high-purity coating with negligible organelle contamination. These results collectively demonstrate that the M-NPs were successfully and authentically coated with mitochondrial membranes, with negligible contamination from other intracellular organelles.

DLS showed that the particle size of M-NPs@CoQ10 remained stable over 7 days in both PBS and 10% FBS media, with no significant aggregation or precipitation. Particularly, the size was maintained at approximately 210–225 nm in PBS and slightly increased to approximately 215–235 nm in 10% FBS, with no significant differences between the two groups (Fig. 1(f)). Regarding the drug delivery capacity, the NPs achieved an encapsulation efficiency (EE) of approximately 80.9% and a drug loading (DL) capacity of 6.0% for CoQ10 (Fig. 1(g)) and *in vitro* release kinetics revealed that both groups exhibited an initial burst release followed by a sustained release phase (Fig. 1(h)). Notably, M-NPs had a more controlled and slower release of CoQ10 compared to the bare NPs group, with approximately 56% cumulative release at 72 h. These results indicate that the mitochondrial membrane coating confers favorable colloidal stability in biological media, effectively resisting serum protein adsorption and aggregation, which lays the groundwork for potential long-term circulating applications *in vivo*.

To investigate mitochondrial internalization, we isolated mitochondria from cells that had been co-incubated with Cy5.5-labeled NPs or M-NPs for 4 h and analyzed them using nano-flow cytometry (Fig. 1(i)). Flow cytometry revealed minimal mitochondrial uptake signals in the control group, whereas the mitochondrial internalization rate was only 8.53% in the NPs group. In contrast, the M-NPs group exhibited significantly enhanced mitochondrial-positive signals, with an internalization rate of 34.9%, which was approximately four-fold higher than that of the NPs group. Quantitative statistical analysis confirmed that the mitochondrial internalization efficiency of the M-NPs was significantly higher than that of the control ($P < 0.0001$) and NPs groups ($P = 0.0001$, Figs. 1(j) and 1(k)). Although the NPs group showed a slight increase compared with the control, the difference was not significant ($P = 0.0051$). These findings demonstrate that the mitochondrial membrane coating endows M-NPs with notable

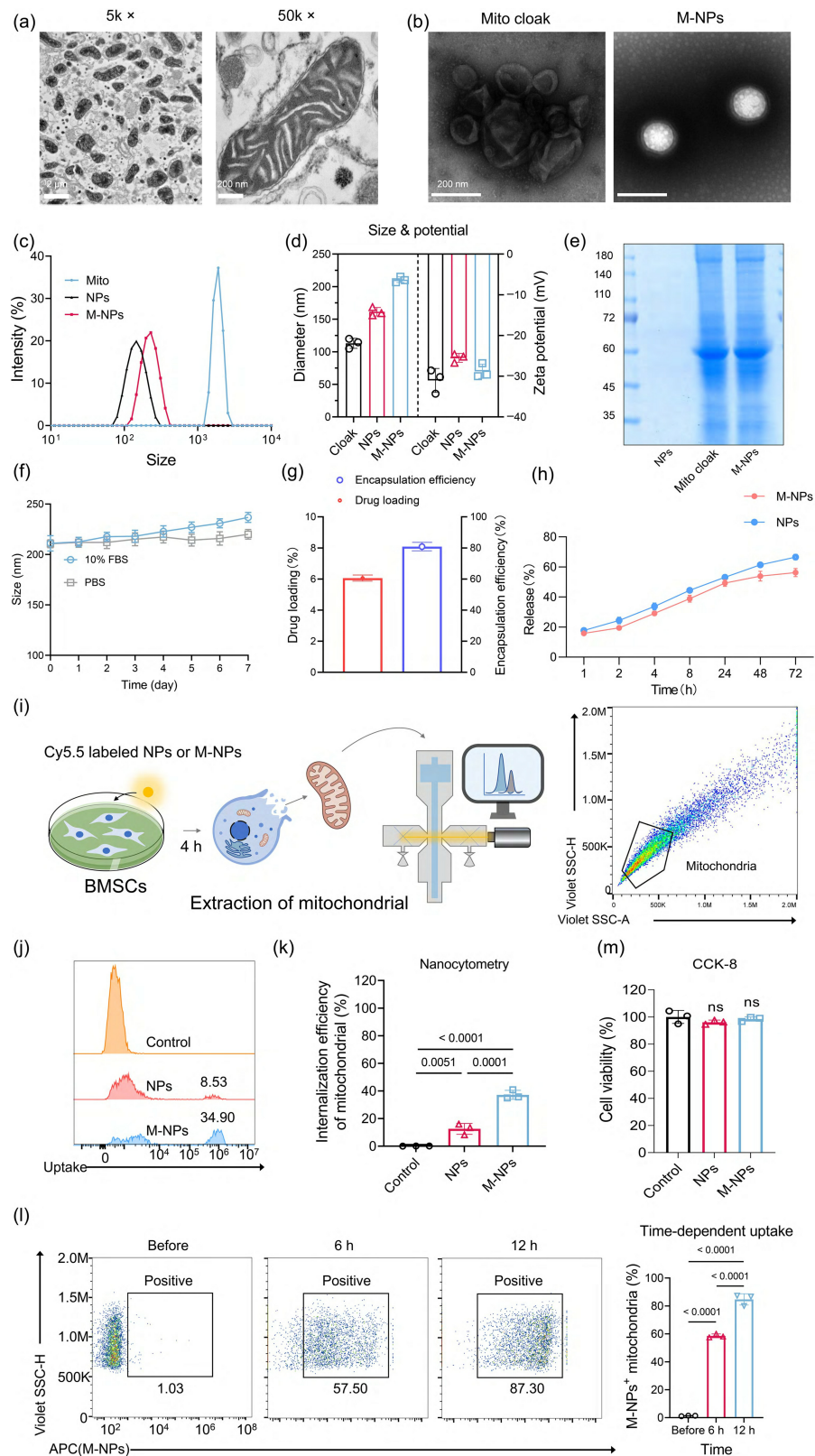


Figure 1 Characterization and mitochondrial targeting efficiency of M-NPs@CoQ10. (a) TEM images of mitochondria at 5000 $\times$ and 50,000 $\times$ magnification. Scale bars: 2 μ m (left) and 200 nm (right). (b) TEM images of Mito cloak and M-NPs. Scale bars: 200 nm. (c) DLS analysis of size distribution for Mito cloak, NPs and M-NPs. (d) Hydrodynamic diameter and zeta potential of Mito cloak, NPs, and M-NPs. (e) Coomassie brilliant blue staining. (f) Stability assessment of M-NPs in PBS and 10% FBS over 7 days. (g) Drug loading (red) and encapsulation efficiency (blue) of the formulated M-NPs@CoQ10. (h) *In vitro* cumulative release profiles of CoQ10 from NPs@CoQ10 (red line) and M-NPs@CoQ10 (blue line) in PBS. (i) Gating strategy for flow cytometric analysis of mitochondria. (j) Flow cytometry histograms showing cellular uptake of NPs and M-NPs. (k) Quantification of panel (j). (l) Time-dependent uptake of M-NPs analyzed using flow cytometry. (m) CCK-8 assay evaluating cytotoxicity of NPs and M-NPs. Data are presented as mean $\pm$ SD.

homologous targeting ability, enabling the efficient delivery of NPs to damaged mitochondria. Further assessment of the cellular uptake behavior of M-NPs using flow cytometry showed that the percentage of M-NPs-positive cells significantly increased over time, from 1.03% before incubation to 57.5% at 6 h, and further to 87.3% at 12 h post-incubation ($P < 0.0001$ for all comparisons), indicating efficient and progressive cellular uptake. The substantial increase in the positive cell population over time suggested an active endocytic process rather than passive surface adsorption (Fig. 1(l)). To further evaluate the specific mitochondrial-targeting capability of the engineered Mito cloak, we used BMSC-derived cell membranes as a control. Following 6 h of incubation with BMSCs, mitochondria were isolated from the recipient cells to quantify the internalized fluorescence intensity via flow cytometry (Fig. S1(b) in the ESM). Representative flow cytometric plots revealed a significantly higher proportion of fluorescence-positive mitochondria in the Mito cloak group compared to the BMSC-membrane group. Quantitatively, the mitochondrial positive rate for the Mito cloak group reached approximately 61.71%, which was nearly 1.8-fold higher than that of the BMSC-membrane group (34.21%, $P = 0.0006$). These data demonstrate that the mitochondrial membrane coating significantly facilitates the precise delivery and accumulation of the nano-cargo within the endogenous mitochondrial network of BMSCs, showcasing superior organelle-targeting specificity over conventional cell membrane coatings.

Additionally, the CCK-8 assay results confirmed that both the NPs and M-NPs exhibited excellent cytocompatibility, with cell viabilities of approximately 95% and 98%, respectively, which were comparable to those of the control group (100%) and showed no statistically significant differences (ns, $P > 0.05$; Fig. 1(m)). Collectively, these results demonstrate that the M-NPs achieve efficient time-dependent cellular uptake while maintaining good biocompatibility and colloidal stability, thus providing a solid foundation for subsequent therapeutic applications.

3.2 M-NPs@CoQ10 protects against oxidative stress-induced mitochondrial dysfunction

CoQ10, a critical component of the mitochondrial electron transport chain, is an indispensable lipophilic electron carrier in the inner mitochondrial membrane and plays an important role in ATP synthesis, effectively promoting mitochondrial recovery. However, the poor water solubility and low bioavailability of CoQ10 in the absence of delivery vehicles hinder its effective cellular permeation and mitochondrial accumulation [19], thereby limiting its clinical application.

To circumvent these limitations, an O/W single-emulsion solvent evaporation method was used in this study to efficiently encapsulate CoQ10 within the core of NPs, followed by sonication and mechanical co-extrusion coating with extracted mitochondrial membranes to construct an M-NPs@CoQ10 biomimetic nanosystem (Fig. 2(a)). To further evaluate the protective effects of M-NPs@CoQ10 on mitochondrial function, we co-cultured BMSCs with H_2O_2 -induced mitochondrial dysfunction in media supplemented with M-NPs, M-NPs@CoQ10, or PBS for 48 h. BMSCs with normal mitochondrial functions were used as controls. We then evaluated and compared mitochondrial function across the different treatment groups. TMRM flow cytometry analysis revealed that H_2O_2 treatment induced a significant decrease in $\Delta\Psi_m$ (Fig. 2(b)). The proportion of hyperpolarized mitochondria

decreased from approximately 75% in the control group to approximately 23%, whereas the proportion of depolarized mitochondria increased from approximately 25% to 75%. The M-NPs-treated group showed limited improvement. In contrast, the M-NPs@CoQ10 treatment group significantly restored the $\Delta\Psi_m$, with the hyperpolarized proportion recovering to approximately 60% ($P = 0.0002$ vs. H_2O_2 group) and depolarized proportion decreasing to approximately 40% ($P = 0.0002$). The mean TMRM fluorescence intensity was also significantly higher in the M-NPs@CoQ10 group compared to that in both the H_2O_2 and M-NPs groups ($P < 0.05$, Fig. 2(c)). Confocal laser scanning microscopy further confirmed these findings, showing that H_2O_2 exposure resulted in a marked attenuation of TMRM fluorescence (red) and an elevation of MitoSOX signals (green), indicating severe mitochondrial depolarization and excessive production of mitochondrial reactive oxygen species (mtROS) (Figs. 2(d) and 2(e)). Quantitative analysis confirmed that M-NPs@CoQ10 treatment significantly enhanced TMRM intensity (Fig. 2(f)) while suppressing mtROS levels (Fig. 2(g)), suggesting a robust antioxidant capacity. Beyond maintaining membrane integrity and redox balance, we investigated the overall health and metabolic activity of the treated cells (Figs. 2(h)–2(j)). H_2O_2 insult significantly impaired mtDNA content, reduced cell viability to approximately 40%, and depleted intracellular ATP levels. Conversely, the administration of M-NPs@CoQ10 substantially recovered mtDNA copy numbers, rescued cell viability, and boosted ATP production ($P < 0.0001$). Collectively, these results demonstrate that M-NPs@CoQ10 effectively protects $\Delta\Psi_m$, maintains mtDNA stability, restores cellular energy metabolism, and enhances cell survival, showcasing excellent mitochondrial-targeted protective function.

This strategy offers three distinct advantages: (1) the NPs significantly enhance the aqueous dispersibility and sustained-release properties of CoQ10; (2) the mitochondrial membrane coating endows NPs with homing-targeting capability, facilitating uptake by damaged cells and accumulation at mitochondria; and (3) the synergistic effect between CoQ10 and mitochondrial membrane components enables simultaneous ROS elimination and $\Delta\Psi_m$ restoration, providing an efficient therapeutic platform for mitochondrial dysfunction.

3.3 M-NPs@CoQ10 promotes osteogenic differentiation

To further evaluate the pro-osteogenic effect of M-NPs@CoQ10, we co-cultured H_2O_2 -treated BMSCs with NPs@CoQ10, M-NPs@CoQ10, or PBS for 48 h. Subsequently, the growth medium was replaced with osteogenic induction medium and the culture was continued for an additional 7 days for subsequent analysis. We first evaluated ALP activity, an early marker of osteoblastic differentiation. ALP staining results revealed that compared to the H_2O_2 group, the number of ALP-positive cells was markedly increased in both the NPs@CoQ10 and M-NPs@CoQ10 groups, with the deepest staining intensity observed in the M-NPs@CoQ10 group, indicating its strongest osteogenic differentiation potential (Fig. 3(a)). Quantitative analysis (Fig. 3(b)) demonstrated that the relative ALP activity in the NPs@CoQ10 and M-NPs@CoQ10 groups was approximately 1.4-fold ($P = 0.0335$) and 1.7-fold ($P = 0.0017$) that of the control group, respectively, with the M-NPs@CoQ10 group having significantly higher ALP activity than that of the NPs@CoQ10 group ($P < 0.05$). To distinguish the respective contributions of the nanocarrier and the drug itself to mitochondrial repair and osteogenesis, we employed flow

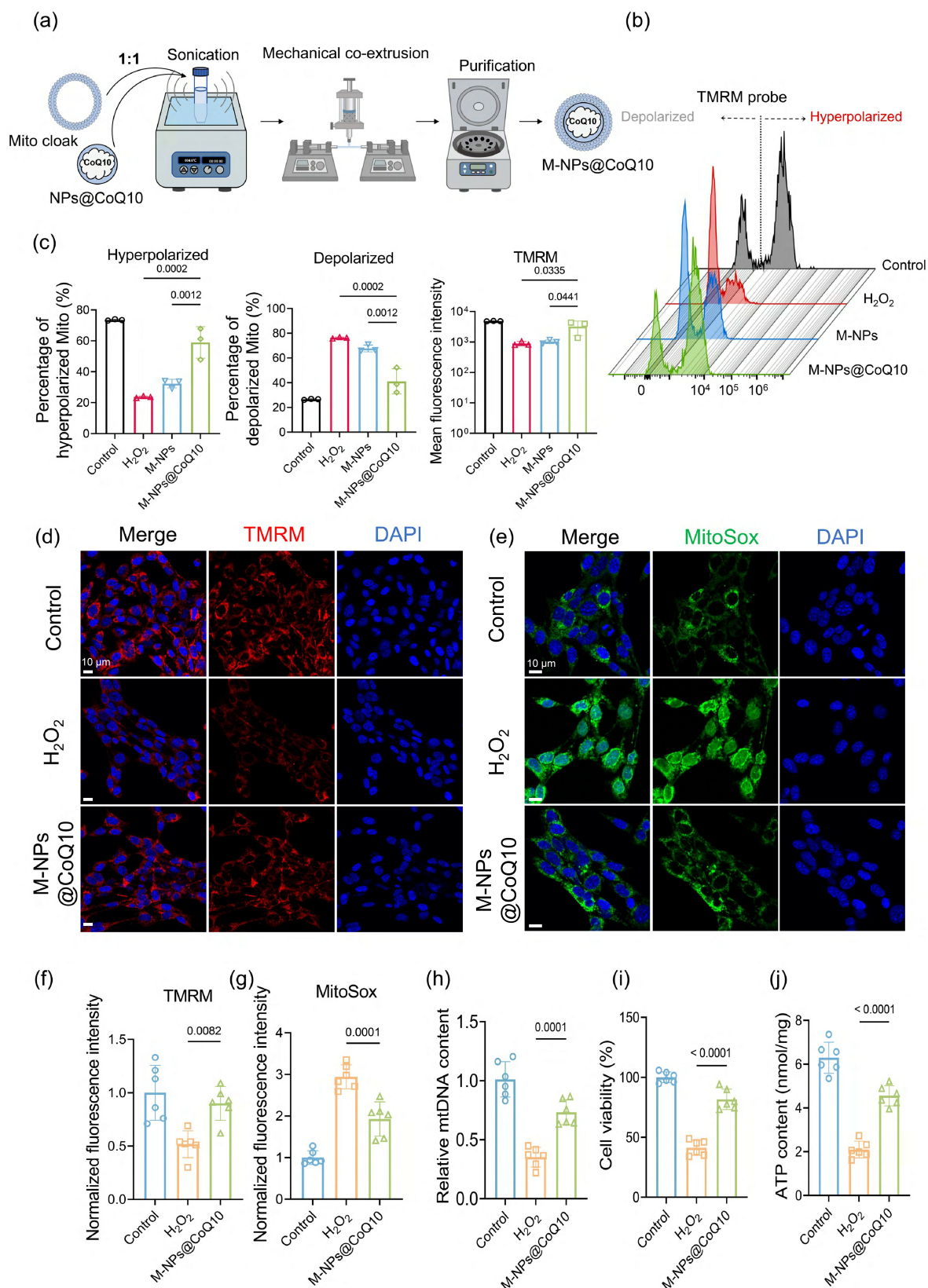


Figure 2 M-NPs@CoQ10 protects against oxidative stress-induced mitochondrial dysfunction. (a) Schematic illustration of M-NPs@CoQ10 nano-system construction. (b) Representative flow cytometry histograms of TMRM staining showing mitochondrial membrane potential. (c) Quantification of hyperpolarized mitochondria percentage, depolarized mitochondria percentage, and mean TMRM fluorescence intensity. Representative confocal microscopy images of cells stained with (d) TMRM (red) for membrane potential and (e) MitoSox (green) for mitochondrial ROS. Nuclei were counterstained with DAPI (blue). Scale bars: 10 μm. Quantitative analysis of normalized fluorescence intensity for (f) TMRM and (g) MitoSox. Quantification of (h) relative mitochondrial DNA content, (i) cell viability, and (j) cellular ATP content. Data are presented as mean ± SD.

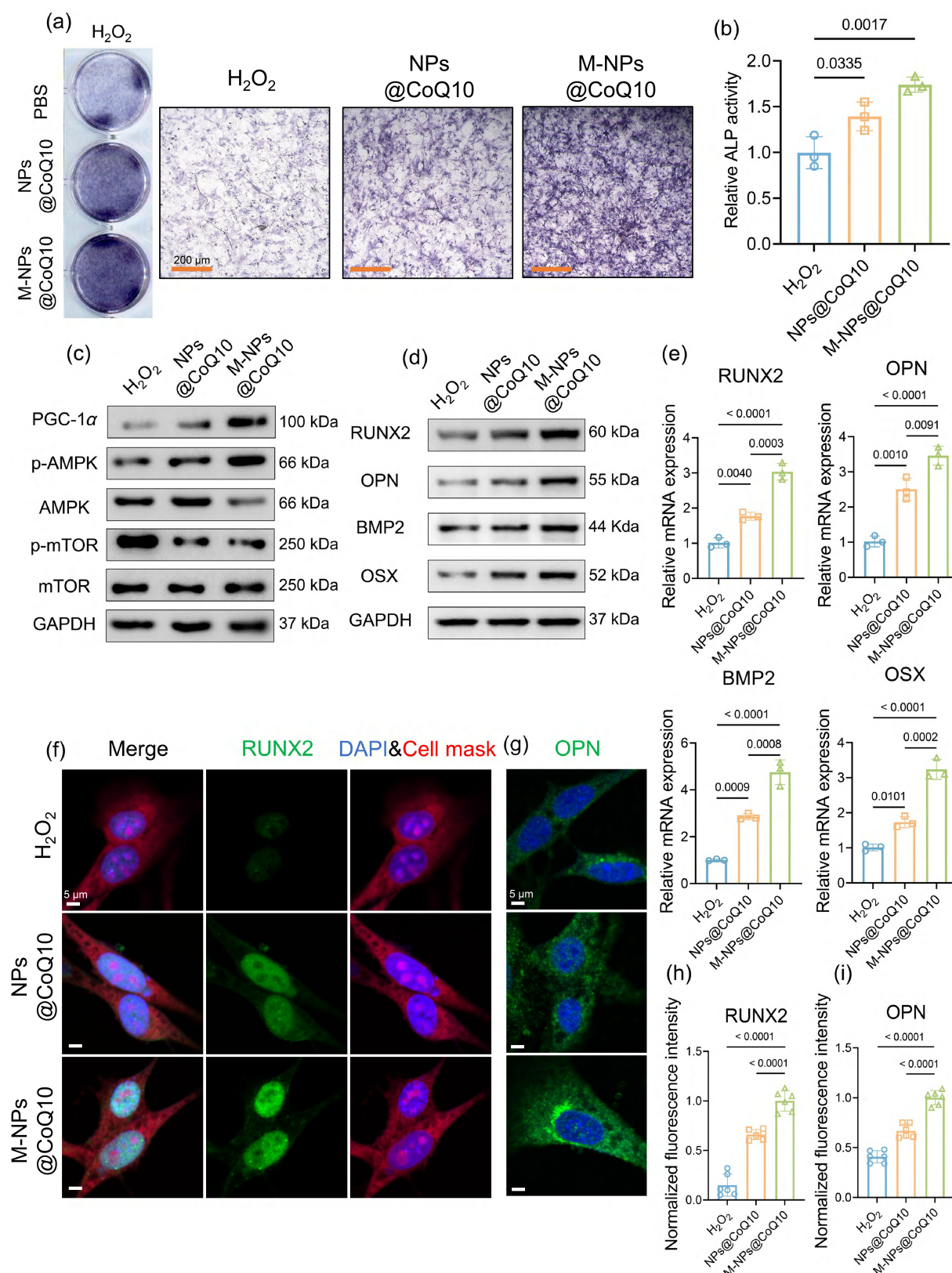


Figure 3 M-NPs@CoQ10 promotes osteogenic differentiation of BMSCs. (a) Representative images of ALP staining showing osteogenic differentiation (left: macro view; right: micro view, scale bars: 200 μ m). (b) Quantification of relative ALP activity of panel (a). (c) Western blot analysis of mitochondrial biogenesis and energy metabolism signaling pathways (PGC-1 α , p-AMPK, AMPK, p-mTOR, and mTOR). (d) Western blot analysis of osteogenic marker proteins (RUNX2, OPN, BMP2, and OSX). (e) Quantification of relative mRNA expression of *Runx2*, *Opn*, *Bmp2*, and *Osx*. (f) Representative immunofluorescence images of the immunofluorescence staining of RUNX2 (green). Scale bars: 5 μ m. (g) Representative immunofluorescence images of OPN (green) expression. Scale bars: 5 μ m. Relative quantitative analysis of the normalized fluorescence intensity for (h) RUNX2 and (i) OPN. Data are presented as mean $\pm$ SD.

cytometry and western blotting (Figs. S1(c) and S1(d) in the ESM), which allowed us to explicitly attribute the enhanced therapeutic efficacy to the synergistic effect of the mitochondrial membrane-coated delivery system.

Given that CoQ10 is a key component of the mitochondrial electron transport chain, we further investigated the impact of M-NPs@CoQ10 on mitochondrial biogenesis and metabolic signaling. Western blot analysis (Fig. 3(c)) revealed that H_2O_2 challenge markedly downregulated the expression of PGC-1 α and suppressed the phosphorylation of AMPK. Notably, M-NPs@CoQ10 treatment effectively reversed these trends, upregulating PGC-1 α and restoring the p-AMPK/AMPK and p-mTOR/mTOR ratios, which indicates improved mitochondrial homeostasis and energy metabolism [20].

The restoration of mitochondrial function was closely associated with enhanced osteogenic signaling. Then we examined the expression of key osteogenic markers, including RUNX2, OPN, BMP2, and OSX, at both protein and mRNA levels. Western blotting (Fig. 3(d)) and RT-qPCR (Fig. 3(e)) results demonstrated that M-NPs@CoQ10 significantly upregulated these markers compared to the H_2O_2 and NPs@CoQ10 groups ($P < 0.001$). Specifically, the mRNA levels of *Bmp2* and *Osx* in the M-NPs@CoQ10 group were approximately 4- and 3-fold higher than those in the H_2O_2 group, respectively.

Furthermore, immunofluorescence staining was performed to visualize the intracellular distribution and expression levels of RUNX2 and OPN. M-NPs@CoQ10-treated cells displayed substantially stronger green fluorescence for RUNX2 (predominantly localized in the nuclei) and OPN (distributed in the cytoplasm) compared to the other groups (Figs. 3(f) and 3(g)). Relative quantitative analysis of the IF intensity (Figs. 3(h) and 3(i)) confirmed that M-NPs@CoQ10 elicited the most significant induction of these markers ($P < 0.0001$). These findings demonstrate that M-NPs@CoQ10 effectively mitigates oxidative stress-induced osteogenic inhibition by rejuvenating mitochondrial function and energy metabolism. The potential osteogenic mechanism involves the activation of the AMPK/mTOR/PGC-1 α signaling pathway: by restoring the phosphorylation of AMPK and mTOR while upregulating PGC-1 α , M-NPs@CoQ10 re-establishes mitochondrial homeostasis. This mitochondrial recovery, in turn, provides the necessary metabolic foundation to drive the expression of key osteogenic downstream effectors, including RUNX2, OPN, BMP2, and OSX. Collectively, our results suggest that M-NPs@CoQ10 promotes bone formation by establishing a robust link between mitochondrial bioenergetics and osteogenic differentiation signaling, demonstrating excellent osteogenic and bone regeneration potential.

3.4 M-NPs@CoQ10 exhibits efficient skeletal accumulation and restores mitochondrial function

To further quantify the systemic distribution and organ-specific affinity of the biomimetic nanoparticles, major organs were harvested for *ex vivo* imaging and quantitative biodistribution analysis at 24 and 48 h post-injection (Fig. 4(a)). Quantitative analysis, expressed as the percentage of injected dose per gram of tissue (%ID/g), revealed that M-NPs distributed across multiple organs, with notable accumulation in the spleen, reflecting typical reticuloendothelial system (RES) clearance [21].

Crucially, M-NPs exhibited a distinct and progressive tropism toward skeletal tissues. As demonstrated, the accumulation in bone

was approximately 40 %ID/g at 24 h, which significantly increased to over 70 %ID/g by 48 h. This time-dependent enrichment in the bone was markedly higher than that observed in other vital organs, including the heart, liver, and kidneys, at the 48 h mark. These results confirm the exceptional bone-targeting efficiency and prolonged retention capability of M-NPs, providing a robust foundation for targeted therapeutic delivery to skeletal sites.

To confirm that the skeletal-accumulated M-NPs could be effectively internalized by resident BMSCs *in vivo*, we performed multi-color flow cytometry analysis on bone marrow cells harvested from mice post-injection (Fig. S1(e) in the ESM). A hierarchical gating strategy was employed to precisely identify the BMSC population: initially, single and viable cells (DAPI⁻) were gated, followed by the exclusion of hematopoietic and myeloid lineages using CD45 and CD11b markers. Within the CD45⁻CD11b⁻ non-hematopoietic fraction, BMSCs were defined as the Sca-1⁺/CD44⁺ subpopulation [22, 23].

Quantitative analysis of the fluorescence signal from DiI-labeled M-NPs within the gated BMSC population revealed a significant shift in the M-NPs group compared to the control group. Representative flow cytometry histograms showed that while the control group exhibited negligible background fluorescence (0.00%), a substantial proportion of BMSCs in the M-NPs group (42.41%) were positive for the nanoparticle signal. Statistical quantification further confirmed that the intake rate of BMSCs in the M-NP treated group was markedly higher than that in the control group ($P < 0.0001$). These findings demonstrate that the biomimetic M-NPs possess superior *in vivo* targeting and cellular entry capabilities, ensuring their efficient delivery into the target BMSC population within the complex bone marrow microenvironment.

Mitochondrial dysfunction plays a pivotal role in delayed bone defect healing [24] and age-related osteoporosis [25], where mitochondrial damage can suppress osteoblast differentiation and bone matrix mineralization. To validate the bone repair efficacy of M-NPs@CoQ10 *in vivo*, we established the bone defect model by creating a full-thickness perforated defect in the mid-shaft of the right femur in mice. Postoperatively, the mice were randomly divided into four groups: the Vehicle (age-matched healthy, non-operated mice), Control (operated mice injected with 200 μ L of PBS), NPs@CoQ10, and M-NPs@CoQ10 group. Starting on the day of surgery, the treatment groups received intravenous injections via the tail vein once a week at a dose of 200 μ L per mouse. All mice were euthanized and samples were collected eight weeks post-intervention (Fig. 4(b)). Consequently, tissue immunofluorescence staining (Fig. 4(c)) and quantitative analysis (Fig. 4(d)) revealed that the fluorescence intensity of SDHA, a marker for the inner mitochondrial membrane, in the bone defect model was significantly reduced in the disease model (Control) group compared to that in the Vehicle group ($P < 0.0001$), suggesting impaired mitochondrial function. Although the NPs@CoQ10 treatment group showed limited improvement, the M-NPs@CoQ10 treatment group exhibited a significant restoration of SDHA fluorescence intensity ($P < 0.0001$ vs. Control group; $P < 0.0001$ vs. NPs@CoQ10 group), which was markedly higher than that in the NPs@CoQ10 group. Real-time quantitative PCR further confirmed that M-NPs@CoQ10 significantly upregulated *Sdha* mRNA expression ($P = 0.0003$ vs. Control group; $P = 0.0164$ vs. NPs@CoQ10 group; Fig. 4(e)). Although M-NPs@CoQ10 demonstrated excellent bone regeneration efficacy in a standard

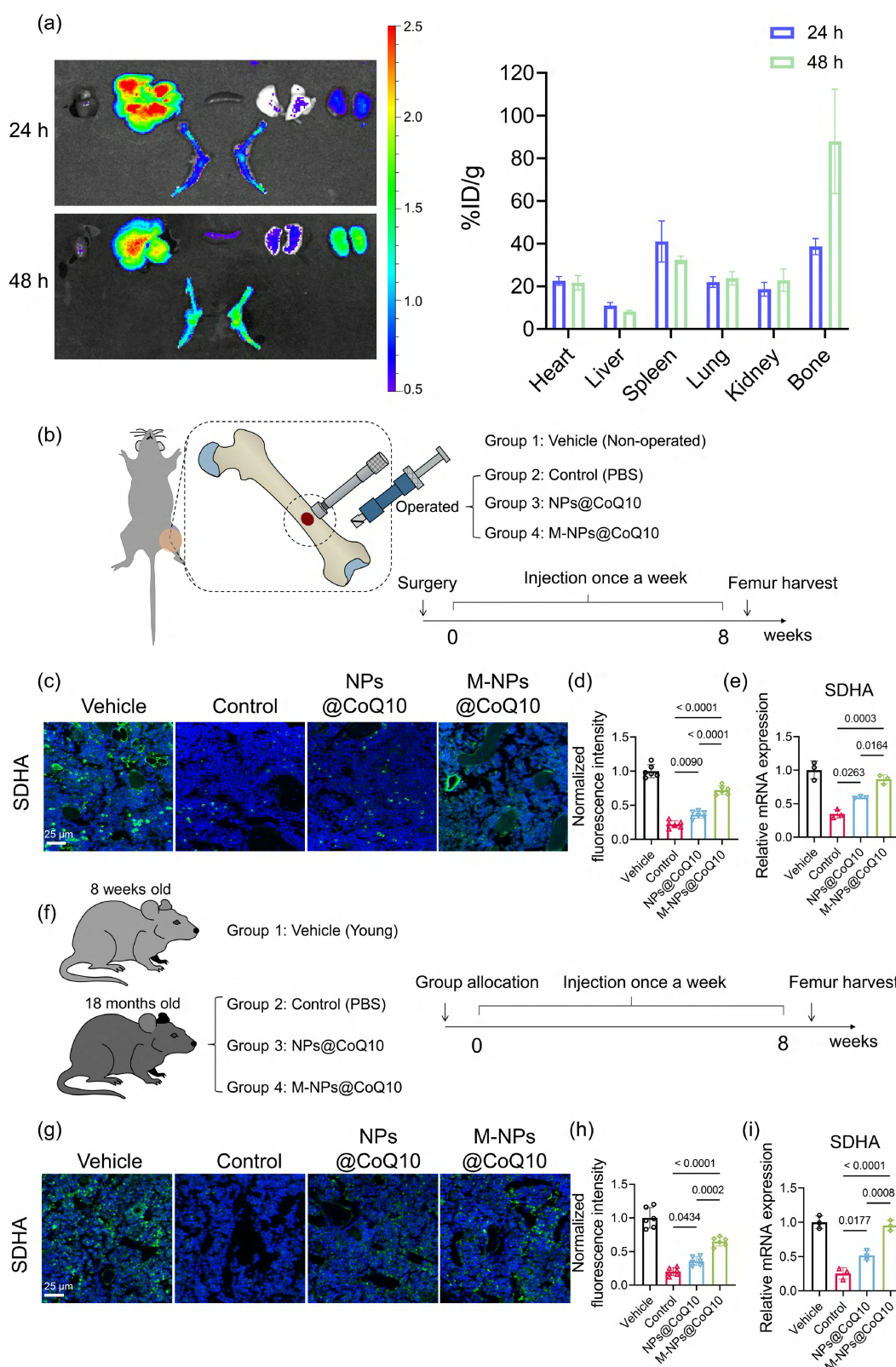


Figure 4 Biodistribution and mitochondrial targeting of M-NPs@CoQ10 *in vivo*. (a) Representative *ex vivo* fluorescence images of major organs (heart, liver, spleen, lung, kidney, and bone) harvested at 24 and 48 h post-injection of M-NPs and quantitative biodistribution of M-NPs in major organs, expressed as the percentage of injected dose per gram of tissue (%ID/g). (b) Schematic illustrations of bone defect model construction. (c) Representative immunofluorescence images of SDHA (green) and DAPI (blue) staining in the bone defect model. (d) Quantification of normalized SDHA fluorescence intensity in panel (c) and (e) relative mRNA expression in a bone defect model. (f) Schematic illustration of osteoporosis model construction. (g) Representative immunofluorescence images of SDHA (green) and DAPI (blue) staining in the osteoporosis model. (h) Quantification of normalized SDHA fluorescence intensity of panel (g) and (i) relative mRNA expression in the osteoporosis model. Data are presented as mean $\pm$ SD.

mouse femoral defect model, patients with clinical bone defects often present with concomitant systemic bone metabolic disorders, such as osteoporosis, where the local microenvironment significantly diverges from that of healthy bone. Osteoporotic conditions are characterized by impaired osteoblast function, hyperactive osteoclast activity, diminished osteogenic differentiation capacity of BMSCs, and severe mitochondrial dysfunction, which can compromise the therapeutic effectiveness of standard nanomaterials. Therefore, to comprehensively evaluate the clinical translational potential of M-NPs@CoQ10, we validated its therapeutic efficacy in a natural age-related osteoporotic mouse model. This allowed us to clarify the osteoinductive capability and mitochondrial function of this biomimetic nanosystem within a pathological bone-loss microenvironment. We established the following groups accordingly: the Vehicle group consisting of 8-week-old healthy male mice serving as a young baseline control; Control group comprising 18-month-old naturally aged mice receiving an equal volume of PBS; and NPs@CoQ10 and M-NPs@CoQ10 groups of 18-month-old mice receiving the corresponding NP treatments. The therapeutic intervention protocol was identical to that used in the bone defect model; mice in the treatment groups received weekly intravenous injections via the tail vein at a dose of 10 mg/kg (CoQ10 equivalent; Fig. 4(f)). The natural age-related osteoporosis model exhibited similar changes (Figs. 4(g)–4(i)). Collectively, these results demonstrated that M-NPs@CoQ10 can effectively target and accumulate at the injury site and restore mitochondrial respiratory chain function, showing excellent *in vivo* therapeutic potential.

3.5 M-NPs@CoQ10 accelerates bone regeneration in trauma-induced bone defects

Bone remodeling dynamics are critical to the overall progression of skeletal repair. We verified its function in promoting mitochondrial recovery by investigating its osteogenic effects *in vivo* through comprehensive and detailed examination. In our established bone defect model, we investigated the *in vivo* therapeutic efficacy of our formulations on bone defects by evaluating the extent of healing using micro-CT. Three-dimensional micro-CT reconstruction (Fig. 5(a)) revealed minimal new bone formation within the defect area in the Control group. The NPs@CoQ10 group exhibited partial bone repair, whereas the M-NPs@CoQ10 group exhibited nearly complete filling of the defect with newly formed bone tissue. Quantitative analysis of the bone regeneration parameters demonstrated that M-NPs@CoQ10 significantly enhanced defect repair. The BV/TV within the defect region was markedly elevated in the M-NPs@CoQ10-treated mice compared with that in the Control ($P < 0.0001$) and NPs@CoQ10 ($P = 0.0001$) groups, reaching levels comparable to those in the Vehicle controls. The Tb.N was significantly increased following M-NPs@CoQ10 treatment compared with that in the Control ($P < 0.0001$) and NPs@CoQ10 ($P = 0.0024$) groups, with values close to those of the Vehicle controls. Notably, the bone mineral density (BMD) of the regenerated tissue was substantially improved in the M-NPs@CoQ10 group compared with that in the Control ($P < 0.0001$) and NPs@CoQ10 ($P = 0.0193$) groups, indicating strictly enhanced mineralization of the newly formed bone (Fig. 5(b)). Immunofluorescence staining and quantitative analysis indicated that the fluorescence intensities of key osteogenic markers (OPN, RUNX2, and OSX) were significantly lower in the Control group than in the Vehicle group ($P < 0.05$), confirming successful

pathological model establishment (Fig. 5(c)). Although the NPs@CoQ10 group showed some recovery in the expression of these markers, the overall effect was limited. In contrast, the M-NPs@CoQ10 group showed significantly upregulated expression levels of OPN ($P < 0.0001$), RUNX2 ($P < 0.0001$), and OSX ($P = 0.0002$), all of which were significantly higher than those in the NPs@CoQ10 group ($P < 0.05$; Fig. 5(d)).

Calcitonin gene-related peptide (CGRP), as a classic marker for sensory nerve fibers, is an important biological indicator of bone regeneration and recovery, and its level changes are closely associated with bone injury, the repair process, and the balance between osteogenesis and osteoclastogenesis [26]. To evaluate the effect of M-NPs@CoQ10 on peripheral nerve regeneration within the bone microenvironment, immunofluorescence staining of CGRP was performed on bone sections from each treatment group (Fig. 5(e)). As demonstrated in the representative images, the Control group exhibited sparse and fragmented CGRP-positive nerve fibers following injury, indicating a compromised neuro-osteogenic niche. While the NPs@CoQ10 treatment showed a slight increase in nerve fiber density, the M-NPs@CoQ10 group displayed significantly more robust and continuous CGRP-positive signaling within the trabecular and cortical regions compared to the other groups. These results suggest that the mitochondrial membrane-coated nanoparticles not only possess superior bone-targeting capabilities but also effectively foster a pro-regenerative environment that facilitates sensory nerve innervation. This enhanced neuro-innervation potentially collaborates with the restored mitochondrial function to synergistically accelerate bone repair and integration. Histological staining corroborated these molecular findings. H&E staining of the M-NPs@CoQ10 group revealed abundant mature bone tissue and well-defined bone marrow cavity structures, whereas Masson's trichrome staining revealed rich collagen fiber deposition (Fig. 5(f)). Conversely, the Control group showed only sparse fibrous connective tissue filling the defects. Furthermore, comprehensive serum biochemical analysis revealed no significant differences in the levels of ALT, AST, BUN, and CK among all groups (ns, $P > 0.05$, Fig. 5(g)) and H&E staining of the major organs (heart, liver, spleen, lungs, and kidneys) demonstrated that no observable tissue damage, inflammatory infiltration, or morphological abnormalities occurred (Fig. S1(f) in the ESM), indicating that M-NPs@CoQ10 has excellent *in vivo* biocompatibility without causing any apparent hepatic/renal impairment or muscle toxicity (Fig. 5(g)). Collectively, these robust results confirmed that M-NPs@CoQ10 effectively promoted *in vivo* bone regeneration while maintaining highly favorable biosafety, providing a safe and effective translational strategy for bone defect therapy.

3.6 M-NPs@CoQ10 promotes bone regeneration in age-related osteoporosis

Osteoporosis is characterized by an imbalance in which bone resorption outpaces formation. Mitochondria are crucial for bone-forming osteoblasts, providing not only ATP for energy, but also biosynthetic precursors for collagen and mineral deposition. Age-related mitochondrial dysfunction leads to bioenergetic failure, impaired matrix synthesis, and ultimately, osteoblast dysfunction. Consequently, insufficient bone formation becomes a pathological hallmark of age-related osteoporosis. Three-dimensional micro-CT reconstruction of the distal femur revealed severe trabecular deterioration in aged Control mice compared with that of young

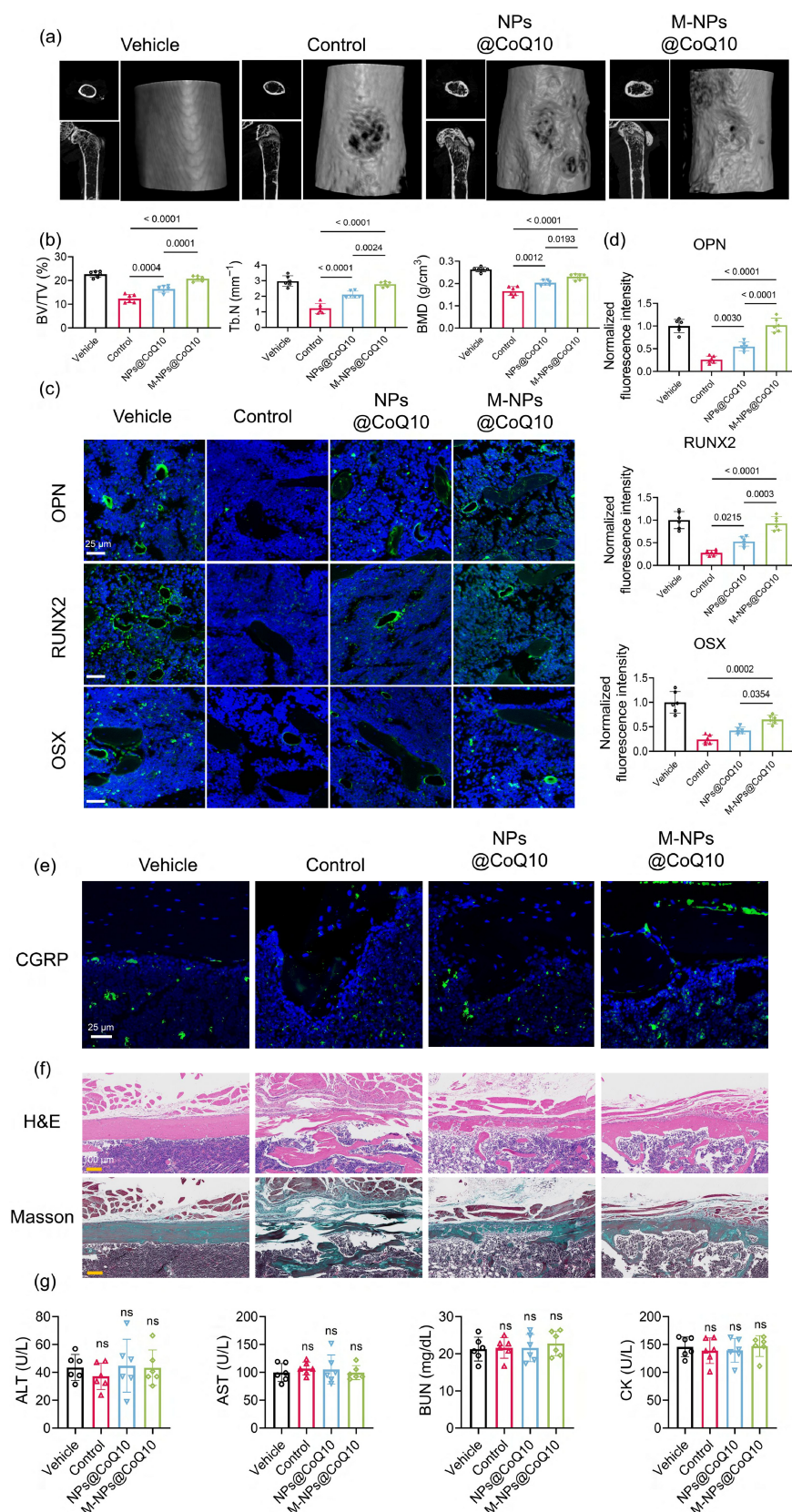


Figure 5 Therapeutic efficacy and biosafety of M-NPs@CoQ10 in the bone defect model. (a) Representative micro-CT three-dimensional reconstructions of distal femurs. (b) Quantitative analysis of trabecular bone parameters including BV/TV, Tb.N, and BMD of panel (a). (c) Representative immunofluorescence images and (d) quantification of osteogenic markers (OPN, RUNX2, and OSX) in femoral sections. Scale bars: 25 μ m. (e) Representative immunofluorescence images of CGRP (green) and DAPI (blue) staining. Scale bars: 25 μ m. (f) Representative histological sections stained with H&E and Masson's trichrome. Scale bars: 100 μ m. (g) Serum biochemical analysis of liver and kidney function markers (ALT, AST, and BUN) and muscle damage indicator (CK). Data are presented as mean $\pm$ SD.

Vehicle-treated animals, characterized by markedly reduced trabecular connectivity and total bone volume (Fig. 6(a)). Notably, treatment with M-NPs@CoQ10 substantially rescued the trabecular bone mass, with architectural parameters reverting to levels similar to those in young mice. In contrast, non-targeted NPs@CoQ10 showed only modest efficacy, whereas Vehicle-treated aged mice exhibited progressive and severe bone loss. Quantitative morphometric analysis clearly demonstrated that aging significantly compromised all trabecular parameters (Fig. 6(b)). Aged Control mice exhibited significantly reduced BV/TV ($P < 0.0001$ versus Vehicle), Tb.N ($P < 0.0001$), and BMD ($P < 0.0001$), strictly concomitant with a massive increase in Tb.Sp ($P < 0.0001$). Treatment with M-NPs@CoQ10 reversed these pathological changes. BV/TV was significantly elevated in M-NPs@CoQ10-treated mice compared with that in both the Vehicle ($P < 0.0001$) and NPs@CoQ10 ($P = 0.0254$) groups, reaching levels comparable to those in young controls. Similarly, Tb.N was significantly increased in the M-NPs@CoQ10 group compared with that in the Vehicle ($P < 0.0001$) and NPs@CoQ10 ($P = 0.0038$) groups, showing no significant difference from young animals. Tb.Sp was significantly reduced in the M-NPs@CoQ10 group, compared with that in the Vehicle ($P < 0.0001$) and NPs@CoQ10 ($P < 0.0001$) groups, indicating heavily restored trabecular connectivity. Furthermore, the BMD substantially improved in the M-NPs@CoQ10 group compared with that in both the Vehicle ($P < 0.0001$) and NPs@CoQ10 ($P < 0.0001$) treatments.

Immunofluorescence staining (Fig. 6(c)) and quantitative analysis (Fig. 6(d)) indicated that the expression of key osteogenic markers (OPN, RUNX2, and OSX) was significantly downregulated in the Control group compared with that in the Vehicle group ($P < 0.05$). Conversely, the M-NPs@CoQ10 group showed a significantly upregulated expression of OPN ($P = 0.0012$), RUNX2 ($P < 0.0001$), and OSX ($P < 0.0001$), all of which were markedly higher than those in the NPs@CoQ10 group.

Sensory innervation is a fundamental component of the neuro-skeletal niche, and its decline is a hallmark of pathological skeletal aging [27]. To evaluate the therapeutic efficacy of M-NPs@CoQ10 in repairing the age-degraded bone microenvironment, we examined the restorative effects on sensory nerve fibers using CGRP immunofluorescence staining (Fig. 6(e)). Quantitative histological analysis revealed a profound loss of CGRP-positive sensory fibers in the aged Control group compared to the young Vehicle mice, signifying a collapsed neuro-skeletal regulatory network in natural senescence. Notably, treatment with M-NPs@CoQ10 demonstrated a potent regenerative capacity, significantly reversing this age-related neural attrition. In contrast to the fragmented and sparse signals in the untreated and NPs@CoQ10 groups, the bone marrow of M-NPs@CoQ10-treated mice exhibited robust, elongated, and densely branched CGRP-positive nerve networks.

Histological staining further corroborated the micro-CT and molecular findings. H&E staining of the M-NPs@CoQ10 group revealed abundant mature bone tissue and marrow cavity structures, whereas Masson's trichrome staining revealed rich and healthy collagen fiber deposition (Fig. 6(f)). In contrast, the Control group exhibited only sparse fibrous connective tissue. Additionally, serum biochemical analysis showed no significant differences in ALT, AST, BUN, and CK levels among all groups (ns, $P > 0.05$), definitively proving that M-NPs@CoQ10 possesses excellent *in vivo* biocompatibility, even in a frail, aging organism (Fig. 6(g)).

Collectively, these robust data confirm that M-NPs@CoQ10 can effectively promote bone regeneration under severe osteoporotic conditions, providing a safe and effective translational strategy for the treatment of osteoporotic bone defects.

4 Conclusions

Skeletal disorders, including trauma-induced bone defects and age-related osteoporosis, are prevalent clinical challenges characterized by impaired osteogenesis, resulting in substantial morbidity and healthcare burdens. Current therapeutic regimens, such as prolonged bisphosphonate administration, often exhibit delayed efficacy and adverse event risks, underscoring the urgent need for innovative interventions that simultaneously modulate bone resorption and formation. In this study, we developed a biomimetic nanosystem by cloaking PLGA NPs loaded with CoQ10 using isolated mitochondrial membranes derived from BMSCs, achieving efficient mitochondrial targeting and functional rescue. Our findings demonstrate that M-NPs@CoQ10 significantly enhances cellular uptake, restores mitochondrial membrane potential, and promotes osteogenic differentiation compared with conventional controls. The superior therapeutic efficacy observed in both bone defect and natural aging-induced osteoporosis models highlights the critical role of mitochondrial membrane-mediated targeting in overcoming the poor solubility and bioavailability of free CoQ10.

The enhanced cellular uptake and mitochondrial localization of M-NPs@CoQ10 can be attributed to the biomimetic membrane coating, which facilitates membrane fusion or receptor-mediated endocytosis pathways specific to mitochondrial components. Restoration of the mitochondrial membrane potential and attenuation of ROS generation effectively protect BMSCs from oxidative stress, preserving their viability and differentiation capacity. Furthermore, M-NPs@CoQ10 treatment upregulates key osteogenic transcription factors, including RUNX2, OPN, BMP2, and OSX, indicating that transcriptional reprogramming integrates metabolic and developmental cues to enhance bone formation. *In vivo*, this mitochondria-targeted approach drives effective bone regeneration by synergistically modulating osteoblast activity and supporting balanced bone turnover, offering distinct advantages over conventional therapies that primarily inhibit resorption without restoring metabolic function. The therapeutic efficacy of M-NPs@CoQ10 in rejuvenating the aged skeletal niche presents a compelling alternative to current clinical standards for bone regeneration. While recombinant human bone morphogenetic protein-2 (rhBMP-2) remains a clinical "gold standard" due to its potent osteoinductive properties, its application is frequently limited by dose-dependent side effects, including severe localized inflammation and the risk of ectopic ossification. In contrast, our biomimetic platform exerts its effects by fundamentally metabolic means—reversing the oxidative-stress-induced "collapse" of the neuro-skeletal niche rather than merely forcing osteoblast differentiation. By targeting the AMPK/mTOR/PGC-1 α signaling axis, M-NPs@CoQ10 facilitates a more physiological restoration of bone homeostasis, which is particularly critical in the context of geriatric osteoporosis where the BMSC population is inherently compromised. When compared to systemic anabolic therapies such as teriparatide (PTH 1-34), which requires daily administration and lacks bone-specific targeting, the M-NPs@CoQ10 system offers significant pharmacokinetic advantages. As demonstrated by our *in vivo* data, the nanoplatform achieves a robust BMSC internalization

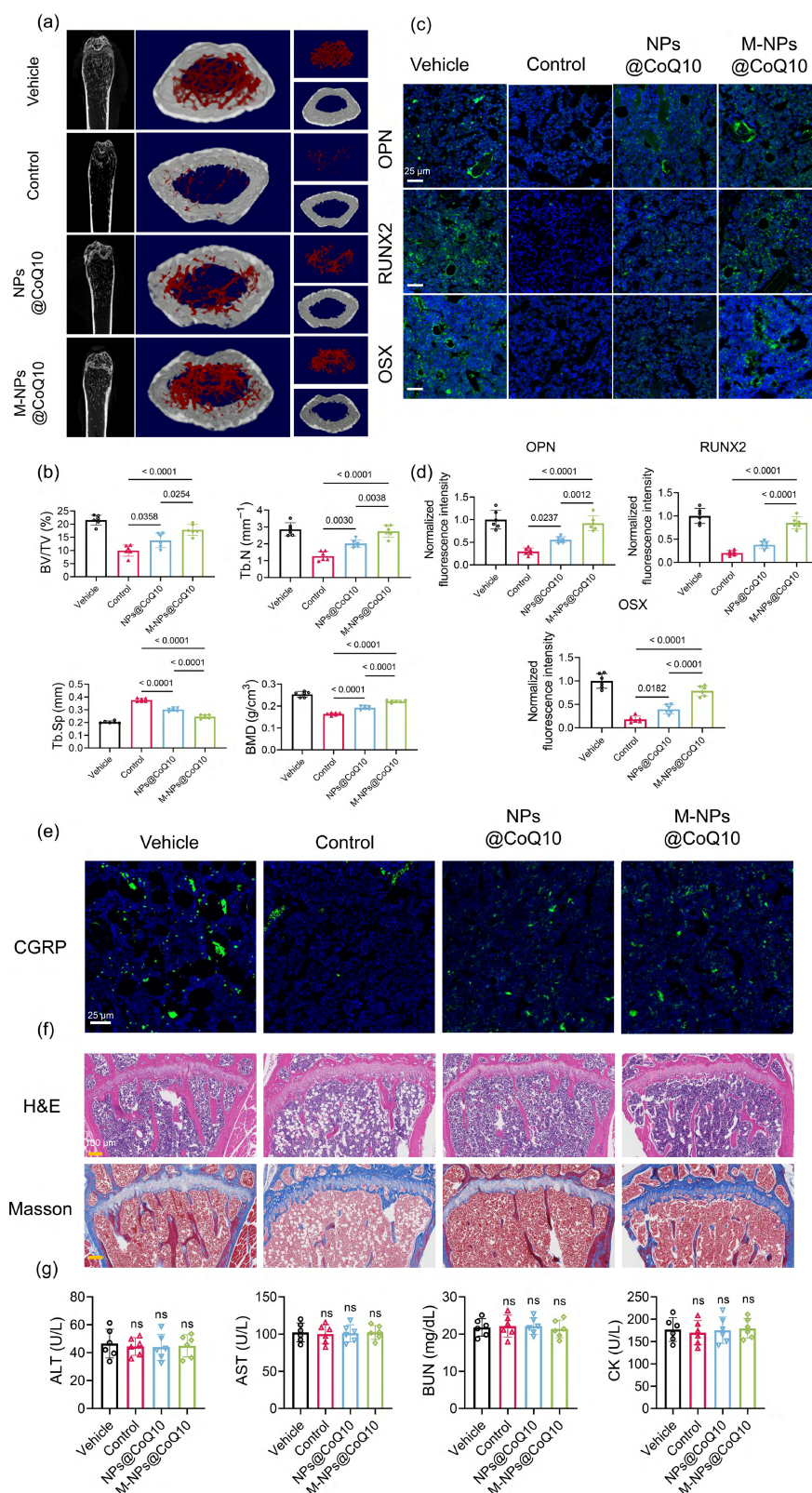


Figure 6 Therapeutic efficacy and biosafety of M-NPs@CoQ10 in a natural age-related osteoporosis model. (a) Representative micro-CT three-dimensional reconstructions of distal femurs showing trabecular bone architecture. (b) Quantitative morphometric analysis of trabecular bone parameters, including BV/TV, Tb.N, Tb.Sp, and BMD of panel (a). (c) Representative immunofluorescence images and (d) quantification of osteogenic markers (OPN, RUNX2, and OSX) in femoral trabecular bone sections. Scale bars: 25 μm. (e) Representative immunofluorescence images of CGRP (green) and DAPI (blue) staining. Scale bars: 25 μm. (f) Representative histological sections stained with H&E and Masson's trichrome showing trabecular microstructure and collagen deposition. Scale bars: 100 μm. (g) Serum biochemical analysis of liver function markers (ALT and AST), kidney function marker (BUN), and muscle damage indicator (CK). Data are presented as mean ± SD.

rate of approximately 42.41% within the bone marrow, whereas systemic small-molecule antioxidants or hormones typically suffer from rapid renal clearance and low skeletal bioavailability. Furthermore, the sustained release profile—showing approximately 55% cumulative release over 72 h—surpasses the transient peaks of standard oral CoQ10 supplements, which have historically failed to reach therapeutic concentrations in bone tissue due to their high lipophilicity. By bridging the gap between high-purity mitochondrial components and targeted antioxidant delivery, this system provides a "bio-smarter" approach that potentially matches the osteogenic output of clinical standards while offering a superior safety profile through the modulation of the endogenous regenerative microenvironment.

To further evaluate the academic significance of M-NPs@CoQ10, it is essential to compare this biomimetic "mito-cloak" strategy with conventional mitochondria-targeted nanoplatforms. Currently, most targeted systems rely on synthetic cationic moieties, such as triphenylphosphine (TPP) or dequalinium (DQA), which utilize the high negative mitochondrial membrane potential ($\Delta\psi_m$) to drive accumulation [28]. While effective, these synthetic ligands often face challenges regarding systemic toxicity and potential interference with the mitochondrial respiratory chain [29]. In contrast, our platform leverages BMSC-derived mitochondrial membranes, which possess inherent membrane-fusion proteins and lipids that facilitate more natural and specific "homing" to recipient mitochondria. This biomimetic inheritance not only enhances delivery efficiency but also circumvents the "accelerated blood clearance (ABC)" effect often observed with purely synthetic surfaces [30].

However, the clinical translation of such cell-derived nanomedicines still faces significant bottlenecks. A primary challenge lies in the standardization of production, as the protein profile of mitochondrial membranes can vary based on the metabolic state and passage number of the source BMSCs, and the shelf-life stability of biological membranes also remains a logistical hurdle compared to purely synthetic carriers. Ensuring the long-term stability of the "mito-cloak" during storage and preventing the loss of delicate membrane proteins remain critical hurdles. Future research should focus on developing optimized lyophilization techniques and investigating the potential immunogenicity of allogeneic mitochondrial components in large-animal models. Additionally, the long-term metabolic fate and potential "off-target" accumulation of mitochondrial membrane components in the liver or spleen necessitate further toxicological investigation. Future research should prioritize the use of standardized, immortalized cell lines for mitochondrial sourcing and investigate lyophilization techniques to enhance the storage stability of this delivery platform, moving it closer to a viable therapeutic product for geriatric osteoporosis. Despite these limitations, the integration of endogenous mitochondrial targeting with potent antioxidants like CoQ10 represents a promising paradigm shift from simple structural replacement to functional bioenergetic restoration in bone regenerative medicine.

Electronic Supplementary Material: Supplementary material (including biosafety detection and *in vivo* targeting of M-NPs) is available in the online version of this article at <https://doi.org/10.26599/NR.2026.94908884>.

Data availability

All data needed to support the conclusions in the paper are presented in the manuscript and the Electronic Supplementary Material. Additional data related to this paper may be requested from the corresponding author upon request.

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Declaration of competing interest

All the contributing authors report no conflict of interests in this work.

Author contribution statement

P. X.: Project administration, methodology. H. S. C.: Data curation, validation, writing of the original draft. K. F. W.: Investigation. H. M. W.: Formal analysis, visualization. Y. F. C.: Completing supplementary experiments for revision. S. Y. S. (Shiyuan Shi): Completing supplementary experiments for revision. Y. M.: Experimental design. Z. W. Z.: Review. S. Y. S. (Shuying Shen): Project administration and resources. Z. J. Z.: Funding acquisition, methodology, writing, review, editing. All the authors have approved the final manuscript.

Informed consent

Not applicable.

Ethics statement

All animal studies were performed according to approved guidelines for the use and care of live animals (Guideline on Administration of Laboratory Animals released in 1988, and 2006 Guideline on Humane Treatment of Laboratory Animals from China). All the experimental procedures were approved by the Committees of Animal Ethics and Experimental Safety of Sir Run Run Shaw Hospital, Zhejiang University (No. SRRSH202107015).

Use of AI statement

None.

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