

Article

Kaempferol Alleviates Glucocorticoid-Induced Osteonecrosis of the Femoral Head by Modulating Macrophage M1/M2 Polarization Through RhoA/ROCK-Mediated Mitophagy Activation

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Abstract

Background/Objectives: Dysregulated macrophage M1/M2 polarization is implicated in glucocorticoid-induced osteonecrosis of the femoral head (GONFH). Reprogramming M1 to M2 macrophages represents a potential therapeutic strategy. Kaempferol (KPF), a natural flavonoid with anti-inflammatory properties, may offer benefits, but its mechanism in GONFH is unknown. **Purpose:** This study aims to explore the therapeutic impact of KPF on GONFH and the mechanisms involved. **Methods:** In vitro, macrophage viability (CCK-8 assay) and polarization (RT-qPCR, flow cytometry) were assessed. Conditioned medium from KPF-treated macrophages was co-cultured with BMSCs and HUVECs to evaluate osteogenic and angiogenic effects. Mechanisms were analyzed using Western blot, immunofluorescence, and flow cytometry. A rat GONFH model validated in vivo effects. **Results:** In vitro experiments revealed that KPF significantly augmented the ratio of M2 macrophages while concurrently diminishing the proportion of M1 macrophages. The conditioned medium derived from macrophages treated with KPF markedly improved the osteogenic and angiogenic capabilities of BMSCs and HUVECs. Immunofluorescence staining and Western blot revealed that KPF regulated macrophage polarization by enhancing mitophagy, which was reversed by the addition of a mitophagy inhibitor. Further experiments confirmed that KPF activated mitophagy by inhibiting the RhoA/ROCK signaling pathway. In vivo, KPF increased the proportion of M2 macrophages and promoted the expression of osteogenic and angiogenic markers. **Conclusions:** In conclusion, our study demonstrates that KPF alleviates GONFH by modulating macrophage M1/M2 polarization through RhoA/ROCK-mediated mitophagy activation. These findings provide novel insights into the treatment of GONFH.

Keywords: kaempferol; mitophagy; RhoA; ROCK; macrophage polarization; GONFH

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1. Introduction

Osteonecrosis of the femoral head is a challenging bone condition primarily caused by excessive use of glucocorticoids. This disease predominantly affects younger populations, with the majority of symptomatic cases eventually requiring surgical management [1]. Without timely intervention, disease progression frequently leads to femoral head collapse in over 50% of patients, resulting in significant disability rates that impose substantial socioeconomic burdens [2]. Consequently, early therapeutic intervention for glucocorticoids-induced osteonecrosis of the femoral head (GONFH) is crucial for halting disease advancement and preserving joint function. Currently, conservative treatments for early-stage GONFH primarily include weight reduction, physical therapy, and anticoagulant or vasodilatory medications, while surgical interventions involve procedures such as core decompression and bone grafting. However, the effectiveness of these approaches remains limited. Therefore, there is a need to develop new pharmacological agents for the treatment of early-stage GONFH to delay the progression of femoral head collapse.

Chronic inflammation is a key factor in the pathogenesis of GONFH, with macrophages playing a crucial role in the inflammatory progression [3,4]. The dynamic crosstalk between bone-resident macrophages and neighboring cells within the microenvironment is fundamental for preserving skeletal homeostasis [5]. Macrophages trigger a chronic inflammatory response through M1 polarization, secreting a range of pro-inflammatory cytokines (such as TNF- α , IL-6, and IL-1 β) and chemokines that contribute to a persistent inflammatory state in GONFH. M2 macrophages, which are alternatively activated and provide anti-inflammatory effects, are mainly found near the blood vessels of the necrotic femoral head. They promote osteogenesis and angiogenesis by locally releasing cytokines such as VEGF, PDGF, BMP-2, and TGF- β [6]. Research has shown that the proportion of M1 macrophages markedly rises in the femoral head specimens from patients with GONFH [7]. Eliminating M1 macrophages in animal models can reduce osteocyte apoptosis, thereby delaying the progression of GONFH [8]. Addressing the imbalance between M1 and M2 macrophages—either by converting M1 macrophages to the M2 phenotype or by preventing M1 macrophage polarization—offers a promising strategy for early intervention in GONFH.

Mitophagy is a fundamental cellular process that enables cells to selectively eliminate damaged or surplus mitochondria via autophagy. It plays a pivotal role in macrophage polarization. It regulates macrophage function by influencing cellular metabolism, inflammation, and the quality of mitochondria [9,10]. Emerging evidence demonstrates that M1 macrophage polarization is associated with the inhibition of mitophagy while activating mitophagy typically promotes the anti-inflammatory M2 phenotype [11–14]. The RhoA/ROCK signaling pathway has been identified as a critical regulator of mitophagy, with mechanistic studies revealing that ROCK inhibitors enhance Parkin translocation to damaged mitochondria [15]. Additionally, this pathway is activated in inflammatory environments, and studies indicate that inhibiting RhoA/ROCK can attenuate inflammation and regulate macrophage polarization [16–18]. These findings collectively suggest that targeted modulation of the RhoA/ROCK-mitophagy axis to promote M2 macrophage polarization may represent a novel therapeutic strategy for inflammatory disorders.

Kaempferol (KPF), a naturally occurring flavonoid abundantly present in numerous dietary sources and medicinal plants, demonstrates diverse pharmacological properties including anti-inflammatory, anticancer, antioxidant, and antidiabetic effects [19,20]. Recent studies have demonstrated that biomimetic nanoparticles loaded with KPF can alleviate the proliferative inflammatory response of macrophages, reduce levels of key pro-inflammatory cytokines, and promote the transition of macrophages from the M1 to the M2 phenotype [21]. Notably, while KPF has been shown to induce mitophagy in neuronal cells, its effects on macrophage mitophagy remain unexplored, and the underlying

molecular mechanisms are yet to be elucidated [22]. To date, no reports have addressed the immunomodulatory effects and mechanisms of KPF in GONFH. Exploring the regulatory effects of KPF on macrophages and understanding its mechanisms may help develop therapeutic strategies for early-stage GONFH.

In this study, we aimed to investigate the therapeutic potential of KPF in attenuating GONFH progression through modulation of macrophage polarization balance and elucidate the underlying molecular mechanisms.

2. Materials and Methods

2.1. *In Vitro* Cell Culture and Drug Treatments

Human Umbilical Vein Endothelial Cells (HUVECs) were sourced from the Cell Bank of the Chinese Academy of Sciences. These cells were grown in an endothelial cell medium (ECM, Gibco, New York, NY, USA). The culture medium was supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 1% penicillin/streptomycin solution (P/S, Service-bio, Wuhan, China), and a 1% addition of endothelial cell growth factor (Gibco, USA). Cell incubation took place at 37 °C within a humid environment maintaining a 5% CO₂ concentration.

Bone Marrow Stem Cells (BMSCs) were extracted using an established method [23]. Under aseptic conditions, BMSCs were harvested by flushing the femurs and tibias. Subsequently, they were cultured in Minimum Essential Medium α (MEM- α , Gibco). This medium contained 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). The isolated BMSCs were then placed into 25 cm² flasks. Incubation occurred at 37 °C within a humid chamber with 5% CO₂. After 24 h, non-adherent cells were discarded. Adherent cells were kept in the complete medium until attaining roughly 80% confluence.

RAW264.7 cells were acquired from the Cell Bank of the Chinese Academy of Sciences. Cultivation utilized Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) containing 10% FBS. Cells were cultured at 37 °C under 5% CO₂ with controlled humidity. Experiments employed cells at passage numbers 3 to 5. Treatment involved co-administration of lipopolysaccharide (LPS, 100 ng/mL, Sigma, Steinheim Am Albuch, Germany) and KPF (Sigma, Germany) for 24 h. Prior to use, KPF was dissolved in dimethyl sulfoxide (DMSO). The compound was then introduced into the culture medium, achieving a 0.1% final DMSO concentration.

2.2. *Cell Viability Assay*

Cell viability following KPF exposure was determined with the Cell Counting Kit-8 (CCK-8) (Beyotime, Shanghai, China) method. Initially, RAW264.7 cells were plated in 96-well plates and allowed to adhere for 24 h. After this attachment period, cultures were treated with KPF at six concentrations (1, 5, 10, 20, 40, and 80 μ M) for a further 24 h interval. Post-treatment viability detection employed the CCK-8 system. All procedures followed the manufacturer's standardized protocols.

2.3. *Flow Cytometric Analysis*

Macrophage polarization (M1/M2 ratio) in RAW264.7 cells was quantified through flow cytometry. Cells were first blocked using PBS containing 5% bovine serum albumin (BSA). Subsequently, they underwent 60 min incubation at 4 °C under dark conditions with two antibodies: PE-labeled anti-mouse CD86 (Biolegend, 105007; 1:100 dilution) and APC-conjugated anti-mouse CD206 (Biolegend, San Diego, CA, USA, 141707; 1:100 dilution). Post-incubation washing was performed with PBS. Cellular analysis employed a flow cytometer (Agilent, Santa Clara, CA, USA). Acquired datasets were processed using NovoExpress software (version 1.6.3, Agilent, USA)

2.4. Real-Time Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-qPCR)

Total RNA isolation from cells employed Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, extracted RNA underwent reverse transcription to generate complementary DNA (cDNA) using a dedicated kit (Accurate Biotechnology, Chongqin, China). Quantitative PCR analysis was then conducted with SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) to examine RNA samples. Primer sequences targeting specific genes (TNF- α , iNOS, Mrc-1, Arg-1, ANG-1, VEGF, ALP, RUNX2) are detailed in Table 1. Finally, relative mRNA expression levels were calculated according to the $2^{-\Delta\Delta C_t}$ methodology.

Table 1. Primer sequences for RT-qPCR.

Gene	Forward (5'-3')	Reverse (5'-3')
iNOS	GGAGTGACGGCAAACATGACT	TCGATGCACAACTGGGTGAAC
TNF- α	CAGGCGGTGCCTATGTCTC	CGATCACCCCGAAGTTCAGTAG
Mrc-1	CTCTGTTTCAGCTATTGGACGC	TGGCACTCCCAAACATAATTTGA
Arg-1	TGGACAGACTAGGAATTGGCA	CCAGTCCGTCAACATCAAACT
ALP	CCAACCTCTTTTGTGCCAGAGA	GGCTACATTGGTGTGAGCTTTT
RUNX2	AGAGTCAGATTACAGATCCCAGG	TGGCTCTTCTTACTGAGAGAGG
VEGF	AGGGCAGAATCATCACGAAGT	AGGGTCTCGATTGGATGGCA
ANG-1	CTCTGCAAAGGGATGCTCCA	GCTCCAGTTGTTGCTTCTGC
β -actin	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT

2.5. Western Blot

Protein expression levels of RhoA, ROCK, PINK1, Parkin, P62, and LC3BII/I were examined through Western blot. First, cells underwent lysis with RIPA buffer (Beyotime, Shanghai, China). Protein concentrations were quantified employing a BCA assay kit (Thermo Fisher Scientific, USA). Next, extracted proteins underwent separation via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The resolved proteins were then electrotransferred onto polyvinylidene difluoride (PVDF) membranes. These membranes were subsequently blocked with 5% skimmed milk. Following blocking, overnight incubation at 4 °C with primary antibodies was performed. After three TBST washes, membranes were exposed to secondary antibodies for 60 min. Chemiluminescent signals were generated using fluorescent substrates. Resultant bands were visualized with a GelView9000 Lite imaging system (BLT Photon Technology, Guangzhou, China). Band intensities were quantified in ImageJ software (version 1.54g). Graphical representations were generated using GraphPad Prism 8. GAPDH served as the loading control. Primary antibodies comprised: anti-GAPDH (Proteintech, Wuhan, China, 60004-1-Ig), anti-PINK1 (Abcam, ab23707), anti-P62 (CST, 5114T), anti-LC3B (Abcam, Waltham, MA, USA, Ab192890), anti-RhoA (CST, 2117T), anti-Parkin (CST, 32833T), and anti-ROCK (Abcam, ab45171).

2.6. RAW264.7 Cells Conditioned Medium Collection

RAW264.7 macrophages were maintained for 24 h under four conditions: PBS, 100 ng/ml LPS, 100 ng/ml LPS + 20 μ M KPF, and 20 μ M KPF. Incubation occurred at 37 °C with 5% CO₂. To ensure complete removal of residual agents, cells received three PBS rinses. Subsequently, macrophages were re-incubated for 24 h in serum-free DMEM at equivalent volume. Post-incubation, conditioned medium was collected following centrifugation at 1000 rpm for 5 min. The acquired supernatant was harvested as a conditioned medium and cryopreserved at −80 °C for subsequent analysis.

2.7. Tube Formation Assay

To assess the angiogenic capabilities of the conditioned medium derived from RAW264.7 cells subjected to various treatments, a tube formation assay was performed in accordance with established methodologies [24]. HUVECs were cultured and treated under different conditions: (1) fresh medium (Control), (2) fresh medium with 20 μ M KPF (KPF), (3) RAW264.7 cells conditioned medium (CM), (4) conditioned medium with 100 ng/ml LPS (CM-LPS), (5) conditioned medium with 20 μ M KPF (CM-KPF), (6) conditioned medium with 100 ng/ml LPS and 20 μ M KPF (CM-LPS + KPF). Initially, HUVECs were cultured in a 6-well plate with different media for 24 h. Simultaneously, Matrigel (Corning, Corning, NY, USA) was thawed overnight on ice after being transferred from -20°C to 4°C . Following this, 50 μ L of Matrigel was utilized to cover a 96-well plate while maintaining the temperature on ice. This was succeeded by an incubation period at 37°C lasting one hour to promote polymerization. Subsequent to rinsing with PBS, HUVECs that had been pre-treated were introduced onto the Matrigel at a density of 2×10^4 cells per well. Following a 12 h incubation period, the medium in the wells was discarded, and the tube formation by HUVECs was visualized using Calcein AM (Corning, USA). Imaging was performed with a fluorescence microscope (Leica, Düsseldorf, Germany) to assess the number of capillary-like structures and loops. Microscopic images were captured from five randomly selected fields within each group, and the total tube length for each group was subsequently quantified using ImageJ software.

2.8. Transwell Migration Assay

HUVECs were cultivated in a six-well plate utilizing different medium for 24 h (Section 2.7). A transwell chamber, characterized by an 8 μ m pore size, was positioned within a 24-well plate. HUVECs were cultivated in a six-well plate utilizing conditioned medium derived from RAW264.7 cells for 24 h. A transwell chamber, characterized by an 8 μ m pore size, was positioned within a 24-well plate. HUVECs were seeded in the upper chamber at 1×10^5 cells per well. The lower compartment contained 600 μ L of 10% FBS. Following 24 h incubation, non-migrated cells in the upper chamber were eliminated. Migrated cells underwent fixation using 4% paraformaldehyde (PFA) for 15 min. Subsequently, these cells were subjected to crystal violet staining for a 15 min period. The number of cells that adhered was then assessed in five randomly selected fields from each experimental group.

2.9. Immunofluorescence Staining

Post-treatment, cells received a single phosphate-buffered saline (PBS) wash prior to immobilization with 4% paraformaldehyde. Permeabilization was subsequently performed employing 0.1% Triton X-100. Following blocking using 5% BSA, samples were incubated with these primary antibodies: anti-LC3B (ab18709, Abcam; 1:100) and anti-Tom20 (11802-1-AP, Proteintech; 1:200). Thereafter, Alexa-fluorophore conjugated secondary antibodies were applied. Imaging was conducted using a confocal microscope (Leica, Germany). For osteocalcin (OCN) immunofluorescence, BMSCs underwent 14-day culture in RAW264.7-conditioned medium. Cellular fixation was conducted with 4% paraformaldehyde (37°C , 15 min). Post-PBS washing, blocking proceeded with PBS containing 5% BSA and 0.5% Triton X-100. Overnight incubation at 4°C with anti-osteocalcin primary antibody (OCN, 23418-1-AP, Proteintech) preceded relevant secondary antibody application. Nuclear counterstaining employed 1 μ g/mL DAPI, with subsequent analysis via laser-scanning confocal microscopy (Leica, Germany).

In vivo, tissue sections were sealed with serum derived from the same species and incubated for one hour. Primary antibody exposure occurred overnight at 4°C with: rabbit anti-CD86 (18704-1-AP), anti-CD206 (13395-1-AP), anti-RUNX2 (20700-1-AP), and

anti-VEGF (19003-1-AP; all Proteintech). After dual PBS washes, appropriate fluorescent secondary antibodies were introduced for 2 h at ambient temperature. Subsequently, tissue sections received DAPI staining (4083S, CST). Protein expression levels were then assessed via laser confocal microscopy.

2.10. Immunohistochemical Staining

Following decalcification and paraffin embedding, femoral heads were sectioned at 5 μm thickness. Subsequently, hematoxylin–eosin (HE) staining was applied to these sections. This enabled evaluation of trabecular architecture and detection of bone cell lacunae.

2.11. ELISA Assay

Following centrifugation of femoral head tissue lysates, protein concentrations were quantified via BCA assay kit (Thermo Fisher Scientific, USA), calibrated against bovine serum albumin (BSA) standards. Separately, IL-1 β and TNF- α levels in femoral head supernatants were measured using commercial ELISA kits (R&D Systems, Minneapolis, MN, USA).

2.12. Animal Experiments

This investigation employed male Sprague-Dawley (SD) rats aged 8–10 weeks. The animals weighed 180–340 g. These subjects were sourced from Xi'an Jiaotong University's Experimental Animal Center (China). Before the experiment, the rats were provided with a standard laboratory diet and were kept under regulated environmental conditions. The GONFH model was established based on previously published protocols [25]. Ethical approval for all procedures was granted by Xi'an Jiaotong University's Animal Ethics Committee (Approval ID: XJTUAE2025-2321). Sixty rats underwent random allocation into three cohorts ($n = 20/\text{group}$) through random number table assignment. A total of sixty rats were randomly assigned to three groups ($n = 20$ per group) using a random number table. The sample size was determined by a priori power analysis based on effect sizes from previous similar studies (power = 0.8, $\alpha = 0.05$). Group allocation and treatment administration were performed by a researcher unaware of the experimental design, and all subsequent imaging, histological processing, and quantitative analyses were conducted under blinded conditions. During the experiment, a total of 8 animals were excluded: 3 deaths occurred in the treatment group, 3 deaths in the model group, and 2 rats in the model group failed to meet the established modeling criteria. The control cohort received equal-volume saline injections. Conversely, other groups were administered 100 $\mu\text{g}/\text{kg}$ LPS via tail vein injection. Subsequently, three intramuscular doses of methylprednisolone (MPS, 40 mg/kg, MCE, Monmouth Junction, NJ, USA) were delivered at 24 h intervals. KPF was initially dissolved in dimethyl sulfoxide (DMSO), then diluted in saline to yield a 2.5 mg/ml final concentration. The KPF cohort received 5.0 mg/kg KPF by twice-daily oral gavage over 14 days. Both control and model groups were given equivalent volumes of PBS.

2.13. Micro-CT Analysis

Following a 5-week rearing period, rats were euthanized for femoral head collection. These were immediately fixed in 4% paraformaldehyde overnight. The femoral head was scanned using a micro-CT scanner (SkyScan 1276, Bruker, Billerica, MA, USA) at a resolution of 10 μm . Three-dimensional reconstructions of the trabecular bone structure were generated using the NRecon software (version 1.7.1.6 Bruker, USA), and bone morphometric analysis was performed with CTAn software (version 1.18.8.0 Bruker, USA). Analysis focused on key microstructural parameters: trabecular thickness (Tb.Th), trabecular

separation (Tb.Sp), bone volume per tissue volume (BV/TV), and trabecular number (Tb.N).

2.14. Statistical Analysis

To ensure reliability, all experiments were performed with at least three biological replicates. Data are presented as mean $\pm$ standard error of the mean (SEM) and statistically analyzed using SPSS 20.0 (IBM, Armonk, NY, USA) and GraphPad Prism 8 (GraphPad Software, Boston, MA, USA). When data met assumptions of normality and homogeneity of variance, differences among groups were assessed by one-way ANOVA, followed by Tukey HSD test for multiple comparisons. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Effects of KPF on the Viability of RAW264.7 Cells

Figure 1A illustrates the chemical structure of KPF. To evaluate the cytotoxic effect of KPF on RAW264.7 cells, CCK8 assay was conducted (Figure 1B). The results showed that KPF concentrations below 40 μ M had no detectable cytotoxicity, while a concentration of 40 μ M resulted in significant cytotoxic effects. Therefore, we chose 20 μ M as the treatment dose.

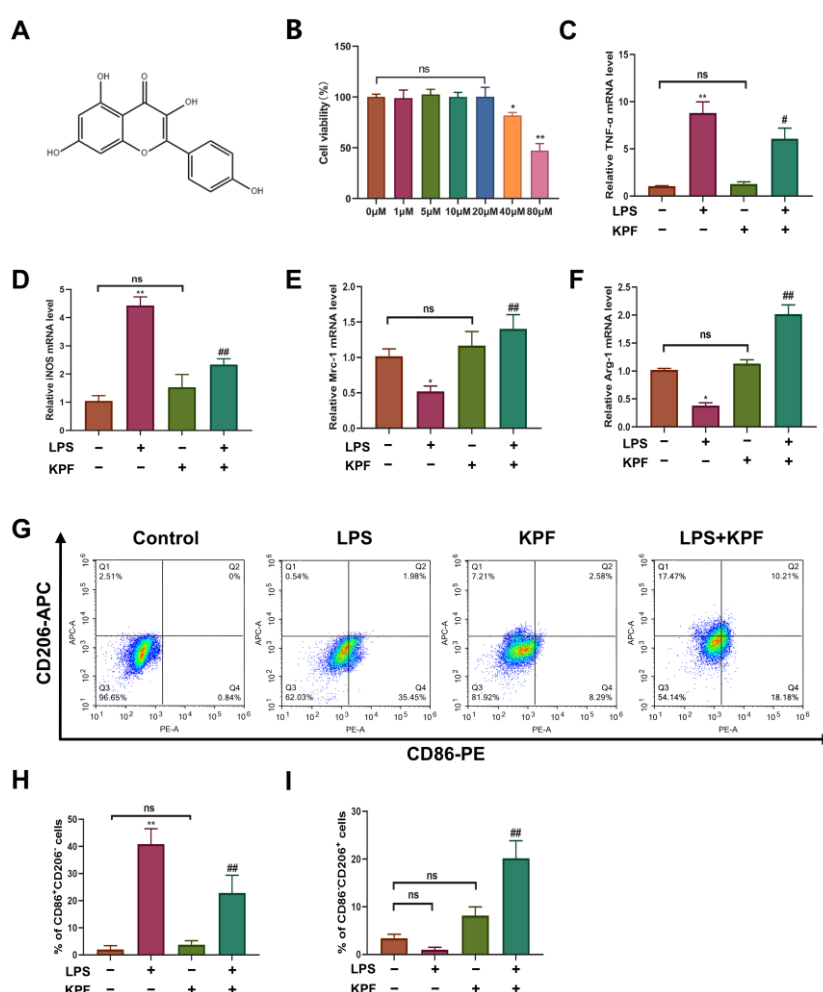


Figure 1. KPF regulated macrophage polarization in vitro. (A) The chemical structural formula of KPF. (B) RAW264.7 cells viability was measured by CCK8 assay after 24 h of treatment with a concentration gradient of KPF, $n = 3$. (C–F) The mRNA levels of M1 polarization markers (iNOS, TNF- α , Mirc-1, Arg-1).

α) and M2 markers (Arg-1, Mrc1) were measured in RAW264.7 cells using RT-qPCR, $n = 3$. (G–I) Flow cytometry quantified M1 (CD86⁺/CD206⁻) and M2 (CD86⁻/CD206⁺) macrophage proportions among different groups. Results are shown as mean $\pm$ SEM, $n = 3$. * $p < 0.05$ and ** $p < 0.01$ versus the control group, # $p < 0.05$ and ## $p < 0.01$ versus the LPS group.

3.2. KPF Promoted the Repolarization of Macrophages from M1 to M2 Phenotype

The expression of TNF- α , iNOS, Mrc-1, and Arg-1 serves as key indicators of macrophage polarization. M1 macrophages exhibit upregulated TNF- α and iNOS but downregulated Mrc-1 and Arg-1, while M2 macrophages display the reverse expression profile. LPS treatment led to a significant rise in TNF- α and iNOS alongside a decline in Mrc-1 and Arg-1 in RAW264.7 cells (Figure 1C–F). This pattern confirms a transition toward M1 polarization when compared to untreated controls. Following KPF treatment, Arg-1 and Mrc-1 levels significantly increase, while TNF- α and iNOS levels significantly decrease. This indicates that macrophages are repolarized from the M1 to the M2 phenotype. Notably, when administered alone, KPF did not produce a substantial enhancement of M2 polarization compared to the control group (Figure 1C–F). Flow cytometry results revealed that LPS treatment markedly increased CD86⁺ cell populations while decreasing CD206⁺ cells relative to controls, confirming LPS-induced M1 polarization. In contrast, KPF administration significantly lowered CD86⁺ cells while elevating CD206⁺ populations, suggesting KPF-mediated M1-to-M2 repolarization (Figure 1G–I).

3.3. KPF-Treated Macrophage Medium Promoted HUVECs Migration and Angiogenesis

To elucidate the immunomodulatory role of KPF in angiogenic effects, a portion of HUVECs was exposed to conditioned medium from different RAW264.7 cell cultures (Section 2.7), while the remaining cells were maintained in fresh medium. Compared to the control group, no significant angiogenic effect was observed when KPF was added to the fresh medium. This result indicates that KPF does not exert its angiogenic effect by acting directly on HUVECs. In Transwell chamber assays, LPS treatment was found to significantly suppress HUVEC migration compared to the CM group. In contrast, adding KPF significantly enhanced the migratory ability of HUVECs (Figure 2D,E). To further evaluate in vitro angiogenesis, a significant angiogenic effect was observed with KPF treatment compared to the CM-LPS group, as confirmed by measuring the tube length formed (Figure 2B,C). However, KPF treatment alone did not significantly enhance HUVECs' angiogenesis and migration compared with the CM group. This suggests that KPF may aid HUVECs' migration by promoting macrophage M1-to-M2 repolarization. The RT-qPCR results indicated a substantial elevation in ANG-1 and VEGF expression in LPS-exposed HUVECs following KPF administration (Figure 2F,G).

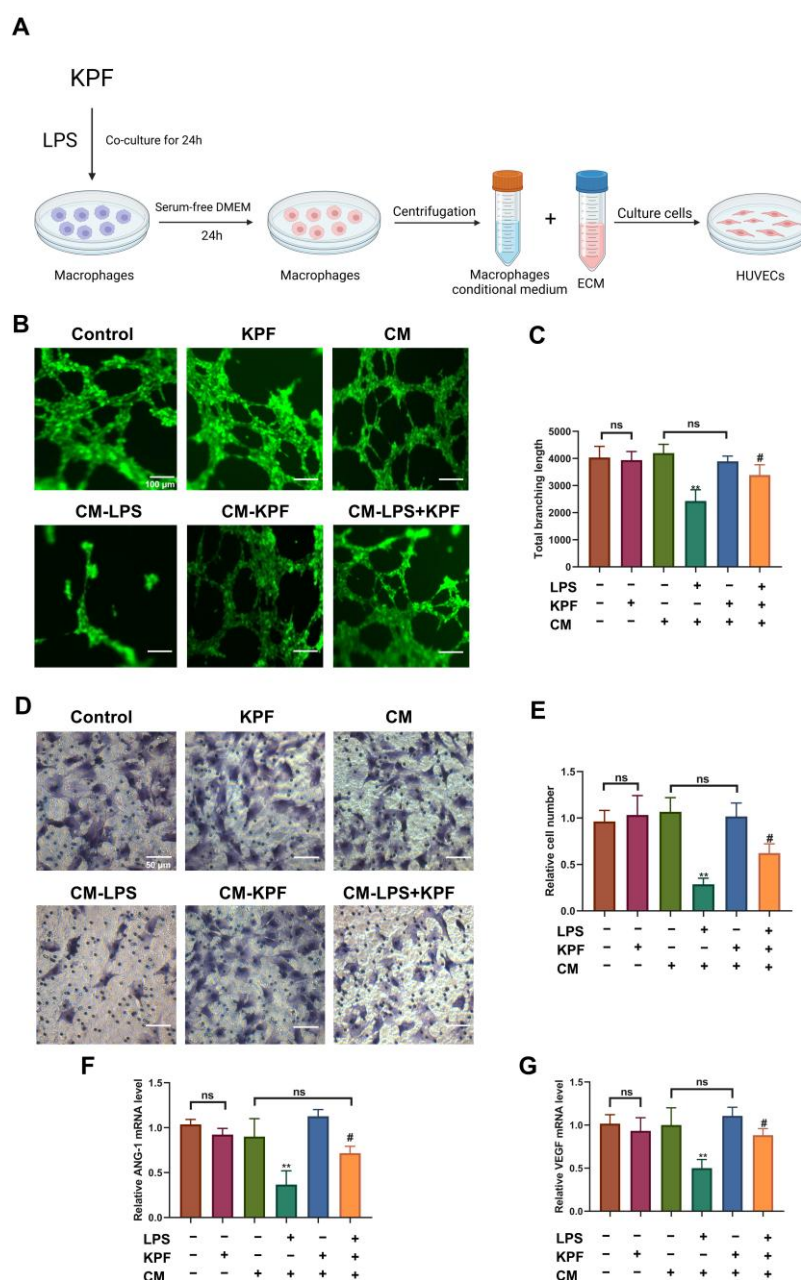


Figure 2. RAW264.7 cells medium treated with KPF promoted the migration and angiogenesis of HUVECs. (A) Schematic diagram of HUVECs angiogenesis and migration using the conditioned medium of RAW 264.7 cells (CM). (B,C) In vitro angiogenesis was evaluated by measuring tube formation in HUVECs cultured with fresh medium and CM for 24 h. Scale bar: 100 μ m, $n = 5$. (D,E) Cellular migratory capacity was assessed using transwell assays. Scale bar: 50 μ m, $n = 5$. (F,G) The expression of the angiogenesis gene of ANG-1 and VEGF was detected by RT-qPCR, $n = 3$. Results were shown as mean $\pm$ SEM, ** $p < 0.01$ versus the CM group, # $p < 0.05$ versus the CM-LPS group.

3.4. KPF-Treated Macrophage-Conditioned Medium Promoted Osteogenic Differentiation of BMSCs

To evaluate the immunomodulatory osteogenic effects of KPF, we prepared RAW264.7 cells conditioned medium. BMSCs were cultured in this conditioned medium for 14 days to assess their osteogenic differentiation ability (Figure 3A). On day 14, we conducted osteocalcin (OCN) immunofluorescence staining. The results revealed a

significant suppression of osteogenic differentiation in BMSCs following LPS exposure compared with untreated controls. However, the downregulation of osteogenic differentiation was substantially reversed by KPF stimulation (Figure 3B,C). Furthermore, the expression levels of osteogenic factors, including ALP and RUNX2, in BMSCs were evaluated via RT-qPCR. KPF significantly increased the mRNA expression of key markers in BMSCs from the LPS group (Figure 3D,E).

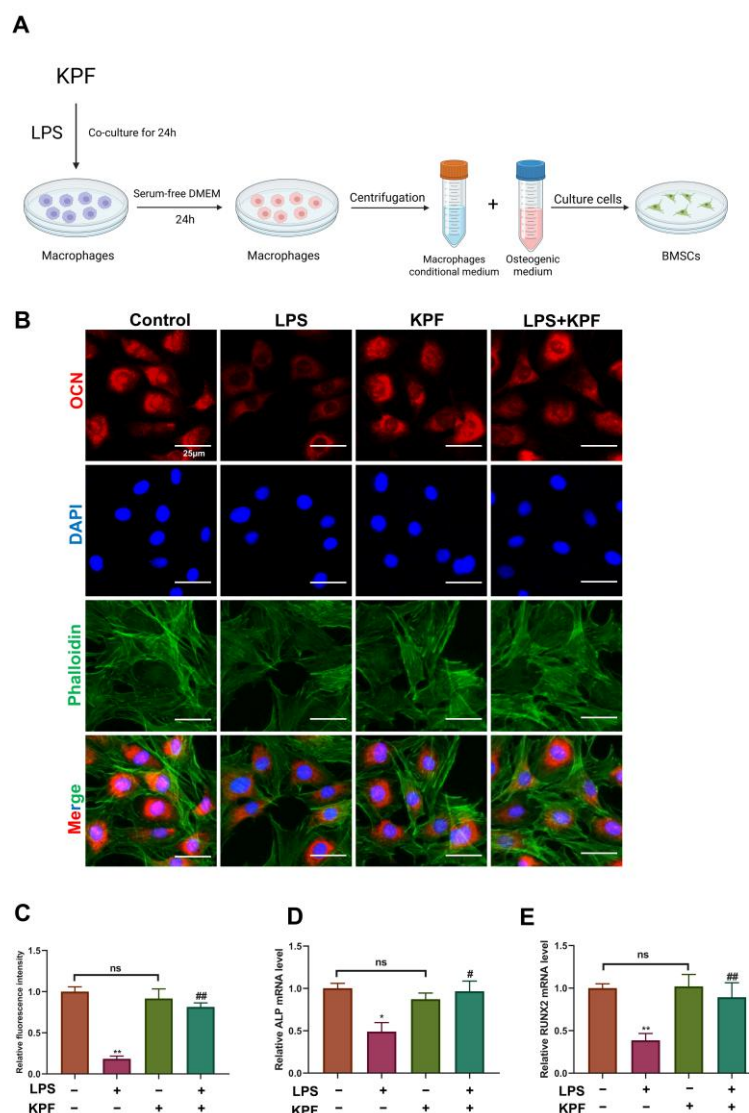


Figure 3. RAW264.7 cells conditioned medium treated with KPF promoted osteogenic differentiation of BMSCs. **(A)** Schematic of osteogenic differentiation using conditioned medium from RAW 264.7 cells cultured with KPF. **(B,C)** The immunofluorescent staining of OCN in different groups after 14 days of cultivation. Scale bar: 25 μ m, $n = 5$ **(D,E)** The osteogenesis gene expression of ALP and RUNX2 was detected by RT-qPCR after 3 days of cultivation, $n = 3$. Results are shown as mean $\pm$ SEM, * $p < 0.05$ and ** $p < 0.01$ versus the control group, # $p < 0.05$ and ## $p < 0.01$ versus the LPS group.

3.5. KPF Enhanced Mitophagy in Macrophages

Studies suggest that KPF can promote mitophagy in nerve cells [22]. However, its role in macrophages remains unknown. Therefore, we utilized Western blotting to examine mitophagy-related protein expression. Data revealed substantial reductions in PINK1, Parkin, and LC3B II/I levels following LPS treatment when compared with untreated

controls. Conversely, the expression of P62 markedly increased, suggesting that LPS effectively impairs mitophagy. However, KPF significantly brought back the levels of those mitophagy proteins (Figure 4A–E). Tom20 is a protein found in the mitochondrial membrane, while LC3B is a protein that makes up autophagosomes in cells. When both proteins are found together, it confirms that mitophagy is happening. Immunofluorescence showed that co-localization of yellow fluorescence in the KPF group went up a lot, further confirming that KPF enhanced mitophagy in macrophages (Figure 4F,G).

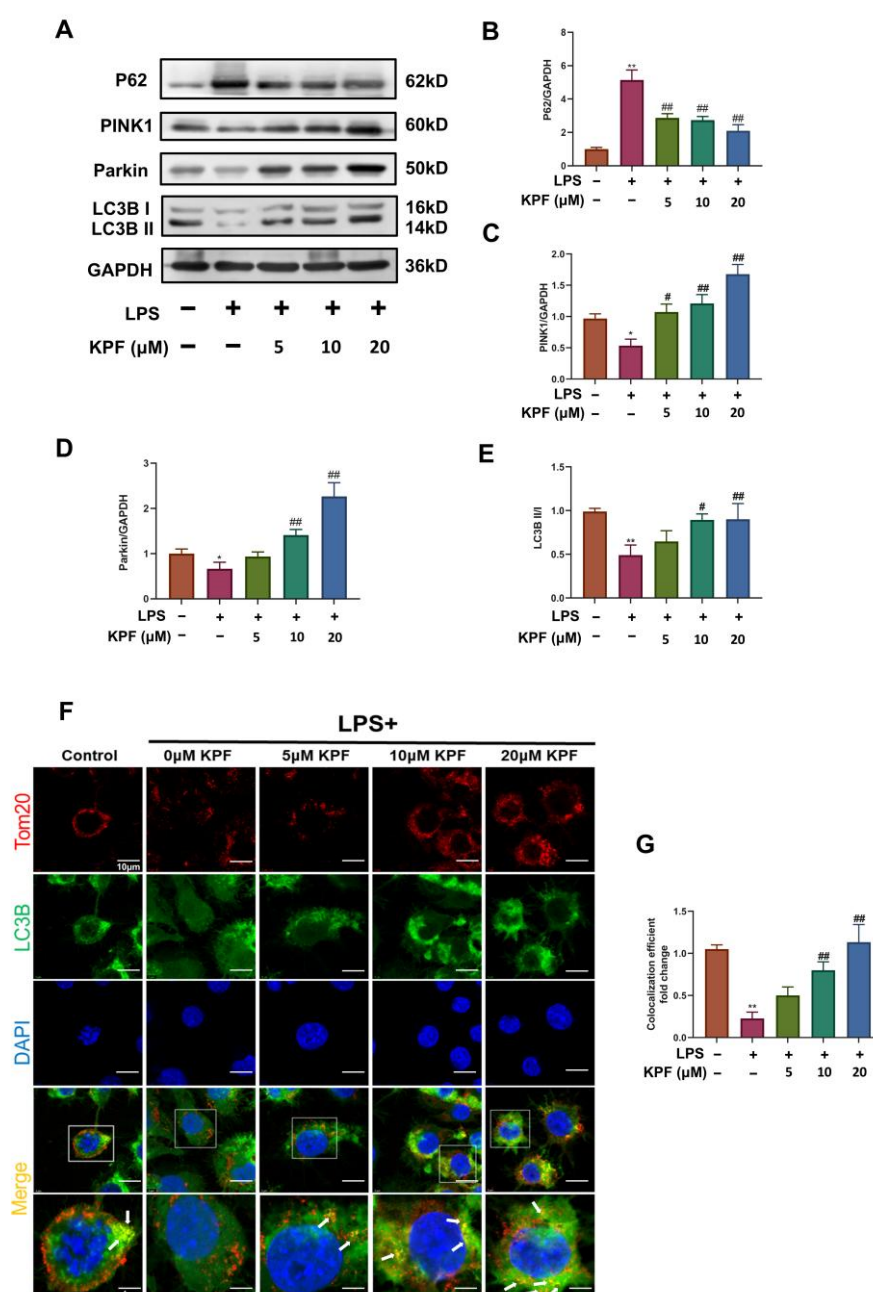


Figure 4. KPF enhanced mitophagy in macrophages. (A–E) Western blot determined the expression of mitophagy-related protein PINK1, Parkin, p62, and LC3B, $n = 3$. (F,G) Fluorescence microscopy images showing colocalization patterns between LC3B (autophagosome maker, green) and Tom20 (mitochondrial marker, red) in RAW264.7 cells. White arrow indicates colocalization of both. Scale bar: 10 μ m, $n = 5$. Results were shown as mean $\pm$ SEM, * $p < 0.05$ and ** $p < 0.01$ versus the control group, # $p < 0.05$ and ## $p < 0.01$ versus the LPS group.

3.6. KPF Promoted Macrophage Re-Polarization M1 to M2 Phenotype Through Mitophagy

Studies indicate a strong connection between mitophagy and macrophage polarization [11–14]. However, it is still unclear if KPF affects macrophage polarization via mitophagy. The results showed that compared with the LPS group, the KPF group exhibited markedly elevated expression of mitophagy-related proteins. In contrast, the effect of KPF was attenuated when combined with the specific mitophagy inhibitor Mdivi-1 (Figure 5A–E), as supported by the immunofluorescence staining results (Figure 5F,G). Additionally, we assessed the M1 (CD86⁺/CD206⁻) and M2 (CD86⁻/CD206⁺) macrophage proportion using flow cytometry. The results indicated that KPF increased the proportion of M2 macrophages in the LPS group while decreasing M1 macrophages. However, adding Mdivi-1 lessened the effect of KPF (Figure 5H–J). These findings demonstrated that KPF promoted macrophage polarization from pro-inflammatory M1 to anti-inflammatory M2 phenotype through mitophagy activation.

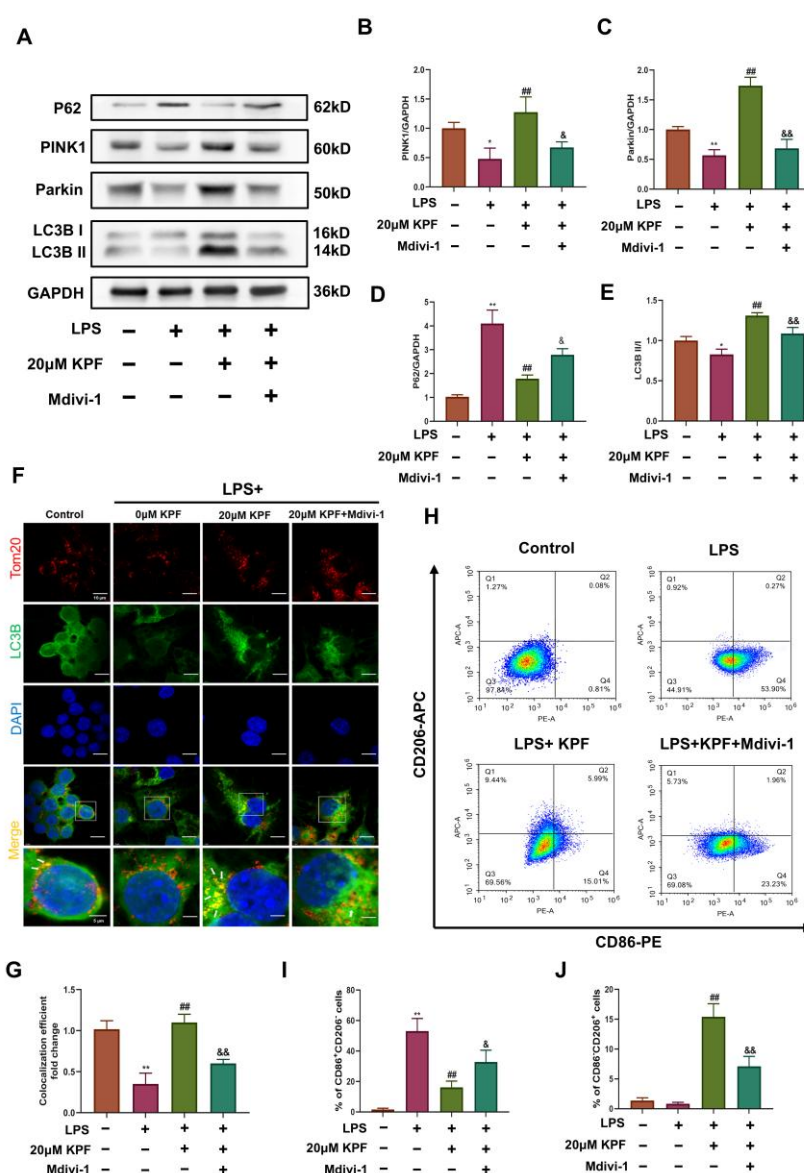


Figure 5. KPF promoted macrophage re-polarization M1 to M2 phenotype through mitophagy. (A–E) RAW264.7 cells were subjected to 100 ng/ml LPS stimulation concurrent with 20 μ M KPF and 50 μ M Mdivi-1 for 24 h. Mitophagy-associated protein expression was assessed through Western blot,

$n = 3$. (F,G) Dual immunofluorescence labeling of LC3B and Tom20 was performed in RAW264.7 cells. White arrow indicates colocalization of both. Scale bar: 10 μm , $n = 5$. (H–J) Flow cytometry analysis was conducted to evaluate the percentages of M1 (CD86⁺/CD206[−]) and M2 (CD206⁺/CD86[−]) macrophage in RAW264.7 cells, $n = 3$. Results shown as mean $\pm$ SEM, * $p < 0.05$ and ** $p < 0.01$ versus control group, ## $p < 0.01$ versus LPS group, & $p < 0.05$ and && $p < 0.05$ versus LPS + KPF group.

3.7. KPF Promoted Mitophagy in Macrophages by Inhibiting the RhoA/ROCK Pathway

The RhoA/ROCK signaling pathway plays a pivotal role in both the process of mitophagy and macrophage polarization [15–18]. To elucidate the impact of KPF on mitophagy within macrophages, Western blot was performed to assess RhoA/ROCK protein expression. Our findings demonstrated significant upregulation of this signaling pathway in LPS-treated macrophages relative to the control group. Notably, the addition of KPF led to a significant attenuation of RhoA/ROCK pathway activity, demonstrating a dose-dependent response with more pronounced inhibition at elevated concentrations of KPF (Figure 6A–C). Western blot analysis further corroborated a significant association between KPF treatment and the modulation of the RhoA/ROCK signaling pathway.

To further confirm that KPF promotes mitophagy in RAW264.7 cells through the RhoA/ROCK pathway, Western blot was performed to evaluate the mitophagy protein expression. The results indicated that KPF treatment significantly upregulated PINK1, Parkin, and LC3BII/I expression while downregulating p62 compared with the LPS control. However, when pentanoic acid (Pa), a ROCK agonist, was added to the LPS group along with KPF, Pa reversed the promoting effect of KPF on mitophagy (Figure 6D–J). Immunofluorescence results demonstrated that KPF enhanced mitophagy in the LPS group; however, this effect was negated by the addition of pentanoic acid (Figure 6K,L). In conclusion, KPF enhances mitophagy in RAW264.7 cells by inhibiting the RhoA/ROCK pathway.

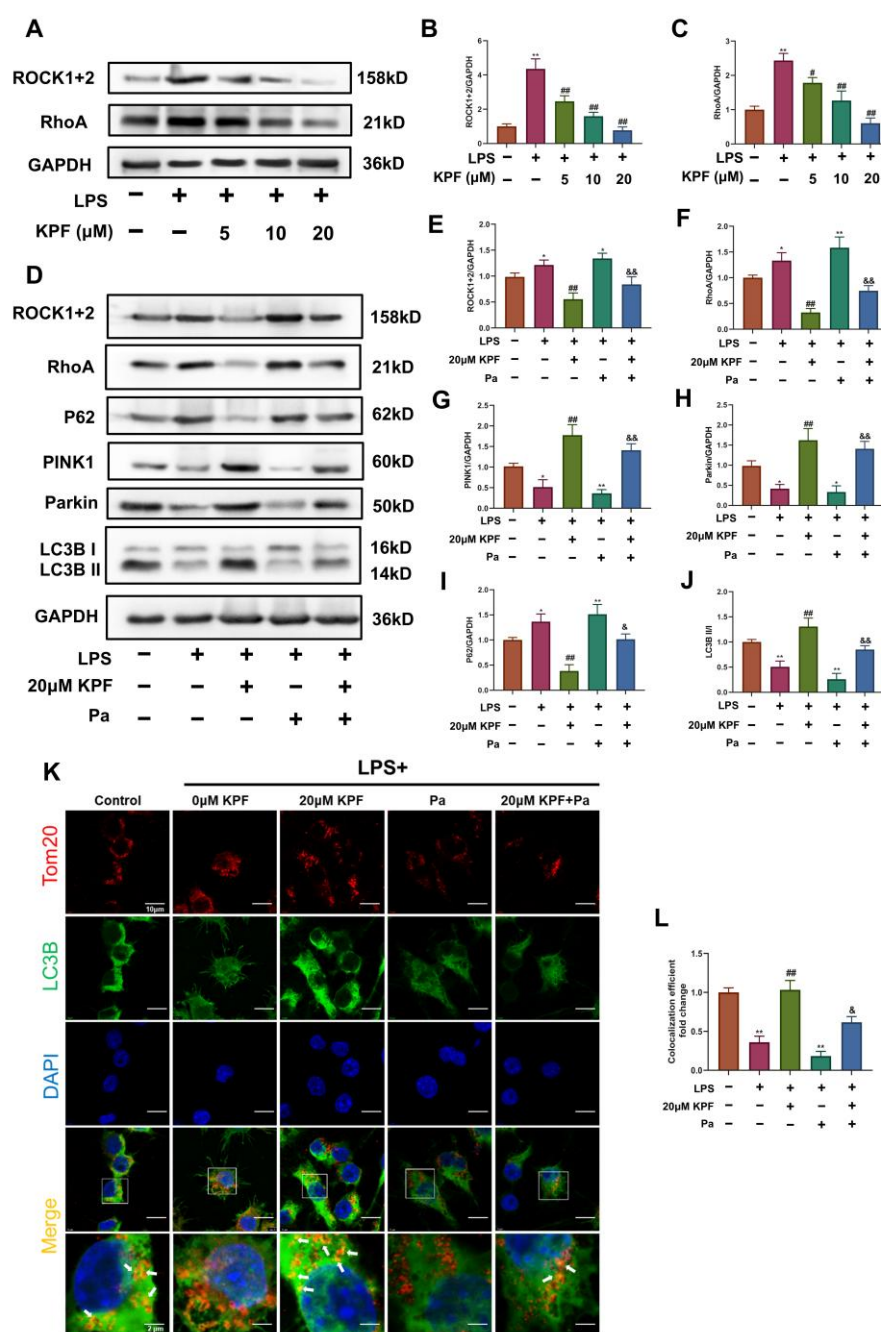


Figure 6. KPF promoted mitophagy in RAW264.7 cells by inhibiting the RhoA/ROCK pathway. (A–E) Western blot determined the expression of RhoA and ROCK in different concentrations of KPF, $n = 3$. (F–J) Western blot analysis on the expressions of RhoA, ROCK, and mitophagy-related protein. Pa, pentanoic acid, ROCK agonist, $n = 3$. (K,L) Immunofluorescent co-staining of LC3B and Tom20 in the RAW264.7 cells. White arrow indicates colocalization of both. Scale bar: 10 μm , $n = 5$. Results shown as mean $\pm$ SEM, * $p < 0.05$ and ** $p < 0.01$ versus control group, # $p < 0.05$ and ## $p < 0.01$ versus LPS group, & $p < 0.05$ and && $p < 0.01$ versus LPS+Pa group.

3.8. KPF Regulated Macrophage Polarization In Vivo

After 5 weeks of treatment, the femoral heads were harvested. We performed immunofluorescence staining on the femoral heads to study how KPF affects macrophage polarization in vivo. In comparison with the control group, the model group demonstrated a markedly elevated presence of M1 macrophages (CD86⁺/CD206⁻) alongside a

significantly diminished presence of M2 macrophages (CD206⁺/CD86⁻) within the femoral heads. However, KPF administration induced macrophage repolarization from pro-inflammatory M1 to anti-inflammatory M2 phenotype (Figure 7B–E). Furthermore, results from the ELISA analysis indicated that KPF treatment led to a significant decrease in the concentrations of TNF- α and IL-1 β in the femoral heads when compared to the model group. (Figure 7C,D).

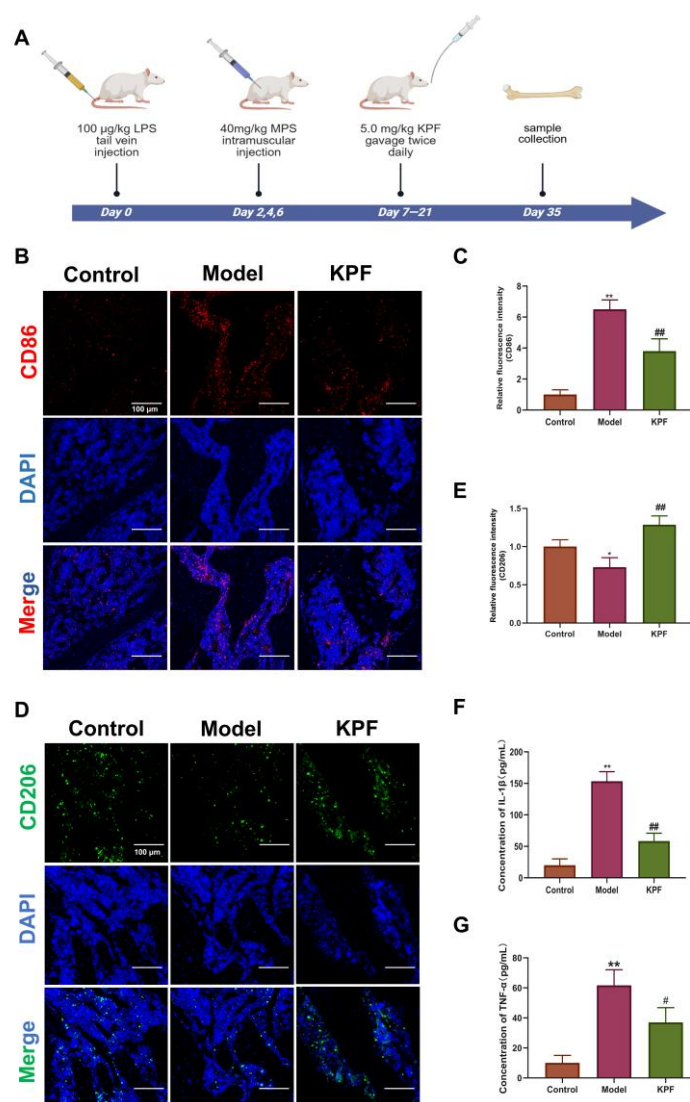


Figure 7. KPF regulated macrophage polarization in vivo. (A) Graphical representation of the experimental protocol for KPF administration in animal models. (B–E) CD86 and CD206 immunofluorescence staining and quantitative analysis were performed in the femoral head. Scale bar: 100 µm, $n = 5$. (F,G) The concentrations of IL-1 β and TNF- α in supernatants of the femoral head were measured in different groups, $n = 3$. Results were shown as mean $\pm$ SEM. * $p < 0.05$ and ** $p < 0.01$ versus control group, # $p < 0.05$ and ## $p < 0.01$ versus model group.

3.9. KPF Promoted Osteogenesis and Angiogenesis In Vivo

We assessed the angiogenic effects of KPF in the femoral heads using VEGF immunofluorescence staining. Findings indicated that the KPF group exhibited a significantly elevated expression of VEGF in comparison with the model group (Figure 8D,E). To evaluate the osteogenic effects of KPF in vivo, we employed micro-CT, RUNX2 immunofluorescence staining, and H&E staining. The micro-CT images demonstrated considerable

trabecular damage within the model group; however, treatment with KPF substantially mitigated this adverse effect (Figure 8B). Moreover, quantitative analyses revealed that the KPF group displayed significant enhancements in various microstructural parameters, including Tb.Th, BV/TV, Tb.N, and Tb.Sp, when compared with the model group (Figure 8C). H&E staining results showed sparse trabeculae and prominent bone cell lacunae changes in the subchondral region of the model group. In contrast, KPF treatment markedly inhibited bone necrosis (Figure 8A). Furthermore, immunofluorescence staining revealed a significant increase in RUNX2 expression following KPF treatment, underscoring its potent osteogenic effects (Figure 8F,G).

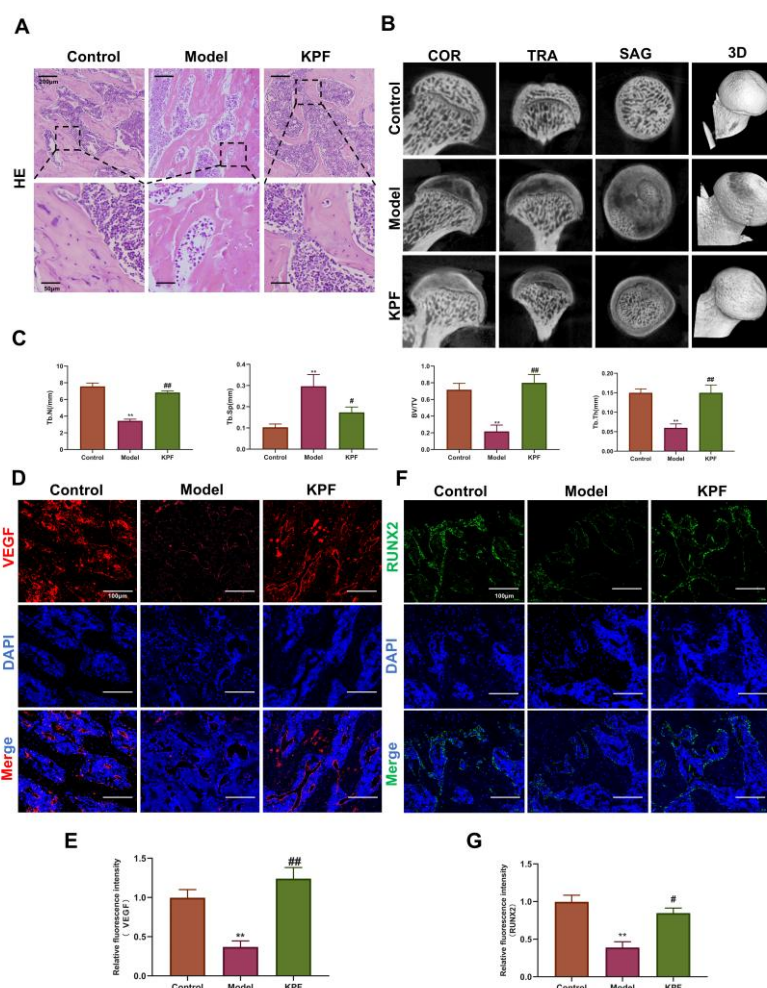


Figure 8. KPF promoted osteogenesis and angiogenesis in vivo. (A) Representative H&E-stained sections of femoral heads in variously treated rat models. Scale bar: 200 μ m (B) Three-dimensional reconstructed images of the femoral head. (C) Quantitative analyses of trabecular separation (Tb.Sp), trabecular thickness (Tb.Th), trabecular number (Tb.N), and bone volume per tissue volume (BV/TV) in the different treatment groups, $n = 5$. (D,E) Immunofluorescence stain of VEGF. Scale bar: 100 μ m, $n = 5$. (F,G) Immunofluorescence stain of RUNX2. Scale bar: 100 μ m, $n = 5$. Results were shown as mean $\pm$ SEM. ** $p < 0.01$ versus control group, # $p < 0.05$ and ## $p < 0.01$ versus model group.

4. Discussion

In recent years, there has been a growing focus on the role of macrophages in modulating the bone microenvironment. This study presents novel findings indicating that KPF mitigates GONFH by regulating macrophage polarization. Mechanistic studies show that KPF promotes mitophagy in macrophages by inhibiting the RhoA/ROCK pathway. This

inhibition promoted macrophage re-polarization M1 to M2 phenotype, which helps regulate immune responses associated with bone formation and blood vessel development. This provides a scientific foundation for using KPF to treat early GONFH (Figure 9).

Accumulating evidence suggests that chronic inflammation in GONFH is associated with an imbalance in macrophage polarization [3,7]. Acute inflammation serves as the preliminary phase in the process of bone regeneration; however, ongoing detrimental stimuli can disrupt the equilibrium of bone homeostasis, thereby leading to chronic inflammatory states. In these prolonged inflammatory environments, necrotic bone undergoes a fibrotic repair process, which obstructs the restoration of normal bone architecture and functionality. There exists a multifaceted interaction between bone tissue and immune cells; immune cells and their products can modulate the activity of osteoclasts, osteoblasts, BMSCs, and osteocytes, thereby linking the immune and skeletal systems [5,6]. Tan et al. reported that inflammation resulting from bone necrosis is accompanied by the accumulation of macrophages in the repair area. In the progressive stage, new blood vessels form in the repair area, with M2 macrophages accumulating around the blood vessels, while M1 macrophages are concentrated in the avascular region [7]. Duan et al. demonstrated that M1 macrophage-derived exosomes impair the balance between osteogenesis and adipogenesis in BMSCs, playing a pathogenic role in GONFH development [26]. In vivo experiments utilizing a macrophage depletion model in rats indicated a significant decrease in TNF- α expression and NF- κ B signaling following the clearance of macrophages, which resulted in diminished cellular apoptosis and a decelerated progression of GONFH [8]. Our research corroborates the involvement of M1 macrophages in GONFH. Compared with the control group, significantly elevated M1 macrophage infiltration in disease model specimens compared to controls. Therapeutic intervention with KPF effectively redirected macrophage polarization toward the M2 phenotype, thereby slowing the advancement of early-stage GONFH.

Mitophagy is a crucial cellular process that regulates mitochondrial homeostasis. It removes damaged mitochondria to prevent harmful substance release and cell death signals, while also regulating mitochondrial quantity to maintain optimal levels. This process is essential for sustaining cellular energy metabolism and balancing oxidative stress [27–29]. Emerging evidence indicates that regulation of mitophagy could represent a promising therapeutic approach for GONFH [30]. Fan et al. found that isovitexin effectively regulates mitophagy by promoting SIRT3 expression, maintaining mitochondrial homeostasis in osteoblasts, inhibiting ferroptosis, and restoring osteogenic capacity, thereby significantly improving GONFH [31]. Zhang et al. revealed that the overexpression of Parkin and the suppression of P53 in BMSCs significantly promoted mitophagy. This modulation led to a decrease in the buildup of impaired mitochondria, notably alleviated apoptosis and senescence in BMSCs induced by stress, and augmented the therapeutic effectiveness of BMSC transplantation in the initial phases of GONFH [32]. Additionally, extensive research has shown a strong link between mitophagy and macrophage polarization. Mitophagy clears damaged mitochondria and reduces reactive oxygen species (ROS) and mitochondrial DNA (mtDNA) leakage, which suppresses M1 macrophage polarization and promotes M2 polarization. Chen et al. found that dimethyl fumarate (DMF) modulates macrophage mitophagy via TUFM, shifting the polarization balance toward M2, preserving mitochondrial function, and suppressing oxidative stress, thereby alleviating periodontitis [12]. Cao et al. reported that paeoniflorin inhibits renal inflammation by enhancing KLF4-mediated mitophagy, promoting macrophage polarization from M1 to M2 [14]. Yang et al. discovered that melatonin enhances AMPK α 2-dependent mitophagy, driving microglial polarization toward the M2 phenotype and mitigating LPS-induced neuroinflammation [13]. In this study, we observed similar findings: in vitro experiments showed

that KPF enhances mitophagy, facilitating macrophage M1-to-M2 polarization. This effect was reversed by the mitophagy inhibitor Mdivi-1.

The RhoA/ROCK signaling pathway has gained significant attention for its role in various physiological processes, such as cytoskeletal rearrangement, inflammation, and cell migration [33]. Research indicates that RhoA/ROCK is closely associated with macrophage polarization. Functional crosstalk exists between RhoA/ROCK and the NF- κ B pathway, as ROCK can phosphorylate I κ B, promoting NF- κ B nuclear translocation and enhancing pro-inflammatory gene transcription [34]. Therefore, inhibiting RhoA/ROCK signaling in inflammatory conditions reduces M1 polarization and promotes M2 polarization. Cai et al. reported that baicalin enhances efferocytosis and modifies macrophage polarization by inhibiting RhoA/ROCK, which facilitates the resolution of inflammation [35]. Zhang et al. demonstrated that parthenolide regulates microglial M2 polarization in cerebral ischemia by inhibiting RhoA/ROCK, alleviating neuroinflammatory damage [36]. Emerging evidence indicates that the RhoA/ROCK signaling axis plays a pivotal regulatory role in mitophagy. Moskal et al. found that ROCK inhibitors promote mitophagy by increasing HK2 (a positive regulator of Parkin) recruitment to mitochondria, enhancing lysosomal targeting and clearance of damaged mitochondria in neurons, thereby ameliorating Parkinson's symptoms [15]. Tu et al. reported that RhoA stabilizes mitochondrial PINK1 by inhibiting its cleavage, facilitating PINK1/Parkin-mediated mitophagy, and protecting against myocardial ischemia [37]. However, whether RhoA/ROCK regulates mitophagy in macrophages remains unclear. In this study, we found that the ROCK agonist suppressed KPF-induced mitophagy, indicating that RhoA/ROCK signaling plays a critical role in mitophagy regulation. In summary, our results show that KPF inhibits the RhoA/ROCK pathway. This inhibition activates PINK1/Parkin-mediated mitophagy, clears damaged mitochondria, and promotes the polarization of macrophages from M1 to M2.

Flavonoids are widely distributed natural products with significant therapeutic potential, including anticancer, anti-inflammatory, antimicrobial, and antioxidant capabilities [38]. Increasing evidence indicates that flavonoids such as quercetin, baicalin, and icariin can modulate the balance of M1/M2 macrophages [39–41]. KPF, a common flavonoid found in tea, apples, and legumes, is used to treat tumors and inflammatory diseases [42,43]. Zhao et al. demonstrated that KPF treatment effectively reprograms macrophage polarization toward the M2 phenotype, conferring anti-inflammatory protection against atherosclerotic plaque formation [21]. Similarly, Chang et al. reported that KPF reduces production of inflammatory cytokines while inhibiting microglial activation, facilitating their phenotypic shift from M1 to M2 and consequently alleviating neuropathic pain [44]. Nonetheless, the mechanism through which KPF promotes the M2 repolarization of macrophages is still unclear, and its role in GONFH has not been studied. Our study demonstrated that KPF promotes macrophage M2 polarization by modulating RhoA/ROCK-mediated mitophagy, which helps alleviate the progression of osteonecrosis through osteoimmunology.

This study has several limitations. First, while RAW264.7 cells were used in this study, incorporating bone marrow-derived macrophages (BMDMs) *in vitro* would strengthen the robustness of the conclusions. In addition, Masson staining was not used to assess the level of bone fibrosis at the pathological level. Second, although we demonstrated that KPF regulates mitophagy via the RhoA/ROCK pathway, the precise mechanism by which KPF interacts with RhoA/ROCK remains unclear and warrants further investigation. Additionally, mitophagy can be classified into two main types: PINK1/Parkin-mediated ubiquitin-dependent pathways and ubiquitin-independent pathways. Whether KPF modulates other forms of mitophagy requires further exploration. Finally, this study focused on the immunomodulatory effects of KPF in bone; however, GONFH

has a multifactorial pathogenesis, involving excessive apoptosis, disrupted lipid metabolism, endothelial dysfunction, oxidative stress, and impaired osteogenic differentiation [1]. Further studies are needed to determine whether KPF ameliorates GONFH progression by targeting these additional pathways. Moreover, as a natural flavonoid compound, kaempferol possesses inherent pharmacokinetic limitations such as poor water solubility, low oral bioavailability, and a restricted ability to accumulate at lesion sites, which pose a significant challenge for its future development into a reliable therapeutic agent. Subsequent efforts could focus on developing corresponding drug delivery platforms to enhance its local sustained-release and targeted effects.

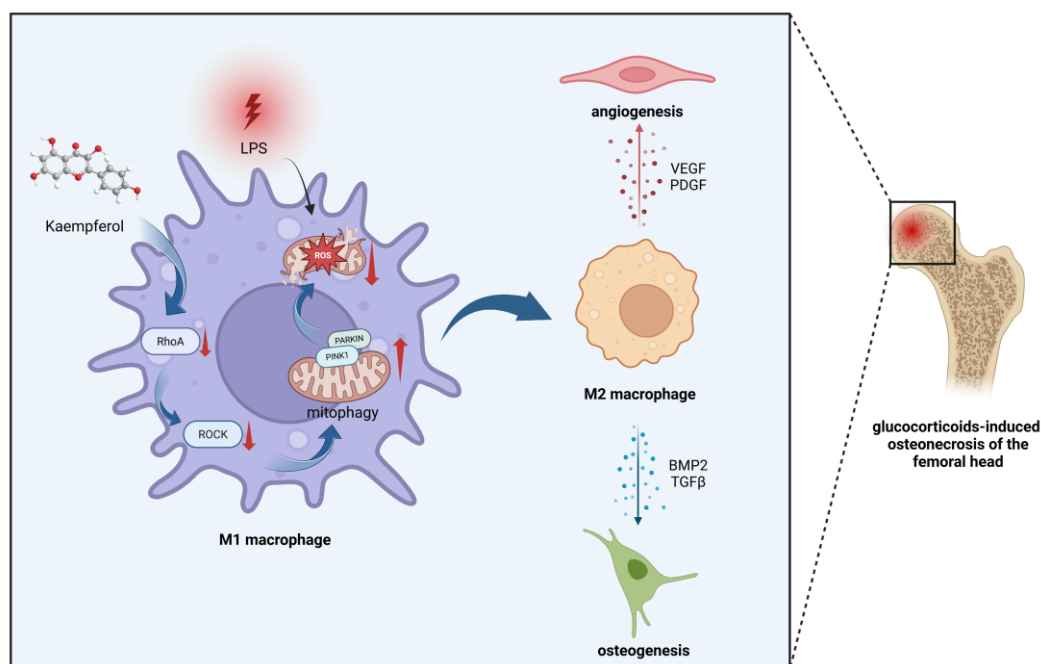


Figure 9. Schematic diagram for the beneficial effects of KPF against GONFH by modulating macrophage M1/M2 polarization through RhoA/ROCK-mediated mitophagy activation.

5. Conclusions

In conclusion, our findings established that KPF mediated macrophage repolarization from M1 to M2 phenotype, resulting in dual osteogenic and angiogenic modulation that alleviated GONFH progression. Mechanistically, KPF potentiates mitophagy by suppressing the RhoA/ROCK pathway, thereby regulating macrophage polarization. These findings provide a novel therapeutic strategy for GONFH treatment.

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Conflicts of Interest: The authors declare no conflicts of interest.

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