

Mechanism by Which Guben Zhuyu Decoction Repairs Impaired Osteogenesis in Glucocorticoid-Induced Osteonecrosis of the Femoral Head: Inhibition of the Mitochondrial Apoptotic Pathway by Targeting the PI3K/AKT Signaling Pathway

Guochen Wang

Changchun University of Chinese Medicine

Yan Zhong

Beijing Shangdi Hospital

Yu Bai

Zhengzhou Orthopedic Hospital

Guoyong Zhang

Zhengzhou Orthopedic Hospital

Chengan Yang

Changchun University of Chinese Medicine

Yuliang Cai

Changchun University of Chinese Medicine

Yi Qu

Changchun University of Chinese Medicine

Xinghua Jiang

Changchun University of Chinese Medicine

Xudong Zhang

Changchun University of Chinese Medicine

Wenjun Cai

Changchun University of Chinese Medicine

Haisi Dong

Changchun University of Chinese Medicine

Wenhai Zhao

The Affiliated Hospital of Changchun University of Chinese Medicine

Changwei Zhao

zCW_1980@126.com

Research Article

Keywords: SIONFH, Mitochondrial apoptotic pathway, Osteoblast apoptosis, PI3K/AKT signaling pathway, Bone remodeling

Posted Date: October 22nd, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10944480/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.
[Read Full License](#)

Additional Declarations: No competing interests reported.

Mechanism by Which Guben Zhuyu Decoction Repairs Impaired Osteogenesis in Glucocorticoid-Induced Osteonecrosis of the Femoral Head: Inhibition of the Mitochondrial Apoptotic Pathway by Targeting the PI3K/AKT Signaling Pathway

Guochen Wang^{1,2}, Yan Zhong^{1,3†}, Yu Bai², Guoyong Zhang², Chengan Yang^{1,4}, Yuliang Cai¹, Yi Qu¹, Xinghua Jiang¹, Xudong Zhang¹, Wenjun Cai¹, Haisi Dong^{1,4*}, Wenhai Zhao^{5*}, Changwei Zhao^{5*}

¹College of Traditional Chinese Medicine, Changchun University of Chinese Medicine, Changchun, Changchun, 130117, China

²Zhengzhou Orthopedic Hospital, Zhengzhou, 450052, China

³Beijing Shangdi Hospital, Beijing, 100085, China

⁴Weifang Hospital of Traditional Chinese Medicine, Weifang, 261041, China

⁵Northeast Asia Institute Research of Traditional Chinese Medicine, Changchun University of Chinese Medicine, Changchun, 130117, China

⁶The Affiliated Hospital of Changchun University of Chinese Medicine, Changchun, 130021, China

*CORRESPONDENCE:

Haisi Dong: donghs@ccucm.edu.cn

Wenhai Zhao: 6177252@163.com

Changwei Zhao: zcw_1980@126.com

†These authors have contributed equally to this work

ABSTRACT

Background: Steroid-induced osteonecrosis of the femoral head (SIONFH) is a highly prevalent disorder characterized by impaired osteogenesis caused by the long-term or high-dose use of corticosteroids. However, currently available effective therapeutic agents remain lacking. Guben Zhuyu Decoction (GBZY), a traditional Chinese medicine formula, has been shown clinically to effectively delay the progression of SIONFH, but its precise therapeutic mechanism remains poorly understood.

Purpose: This study aimed to investigate the mechanism by which GBZY repairs impaired osteogenesis in SIONFH by targeting the PI3K/AKT signaling pathway to inhibit the mitochondrial apoptotic pathway.

Methods: A rat model of SIONFH was established using lipopolysaccharide (LPS)

and methylprednisolone (MPS) to simulate the pathological process in vivo. The therapeutic effects of GBZY on SIONFH were evaluated through behavioral assessments, imaging analysis, histopathological examination, and detection of osteogenic markers. An in vitro model of osteogenesis inhibition was established in MC3T3-E1 cells using dexamethasone. The ability of GBZY to promote osteogenic differentiation was assessed by EdU staining, ALP staining, mineralized nodule staining, and analysis of the expression of osteogenic differentiation markers. Key active components and potential signaling pathways of GBZY were identified by combining HPLC–MS/MS with network pharmacology and were subsequently validated by immunohistochemistry (IHC) and western blotting (WB). Indicators including reactive oxygen species, apoptosis, and mitochondrial membrane potential were measured to evaluate the effects of GBZY on the mitochondrial apoptotic pathway.

Results: In vivo experiments showed that GBZY significantly improved gait, femoral head architecture, and bone microstructure in rats with SIONFH, while upregulating the expression of osteogenic differentiation markers, including ALP, OPN, OCN, BMP-2, and RUNX2. Cell experiments supported these in vivo observations by showing that GBZY enhanced MC3T3-E1 cell proliferation and restored osteogenic differentiation and mineral deposition under dexamethasone stress. HPLC–MS/MS and network pharmacology analyses revealed that tanshinone IIA, kaempferol, quercetin, β -sitosterol, and luteolin were the key active components of GBZY involved in the intervention of SIONFH. In vitro experiments showed that GBZY protected MC3T3-E1 cells from apoptosis triggered by the mitochondrial apoptotic pathway by reducing reactive oxygen species (ROS), increasing BCL-2 expression, decreasing BAX expression, and restoring membrane potential. Further studies confirmed that GBZY activated the PI3K/AKT pathway by increasing the phosphorylation levels of downstream proteins. Notably, the PI3K/AKT pathway inhibitor LY294002 completely reversed all of the abovementioned effects.

Conclusion: This study demonstrated that GBZY promotes osteogenesis by activating the PI3K/AKT signaling pathway, thereby counteracting DEX-induced activation of the mitochondrial apoptotic pathway. These findings provide experimental evidence supporting GBZY as a potential therapeutic strategy for SIONFH.

Keywords: SIONFH; Mitochondrial apoptotic pathway; Osteoblast apoptosis; PI3K/AKT signaling pathway; Bone remodeling

1. Introduction

Steroid-induced osteonecrosis of the femoral head (SIONFH) is an aseptic, necrotic disorder that progresses to progressive hip joint disability **Error! Reference source not found..** Long-term or high-dose exposure to glucocorticoids (GCs) has emerged as the dominant factor behind the disorder **Error! Reference source not found..** A meta-analysis involving 23,561 patients revealed that every additional 10 mg of prednisone taken daily increased the incidence of osteonecrosis by 3.6% **Error! Reference source not found..** Another prospective study involving different

underlying diseases treated with GCs showed that adolescents and adults were at a higher risk of developing SIONFH after steroid use, with an increasingly evident trend toward younger age at onset[4]. Without timely and effective intervention, the femoral head collapse rate in patients with SIONFH can be as high as 80% within 2 years, seriously reducing daily function and increasing both medical and socioeconomic burden.[5][6]. However, current first-line pharmacological treatments for SIONFH still mainly rely on calcium supplements and antiresorptive agents, such as alendronate sodium, and targeted therapies capable of effectively promoting osteogenesis and repairing bone structure remain lacking[7][8].

The pathogenesis of SIONFH is complex, and osteoblast apoptosis with the consequent impairment of bone formation has attracted increasing research attention. Studies have shown that GCs increase osteoblast apoptosis, leading to typical manifestations of suppressed bone formation, including empty osteocytic lacunae, reduced bone tissue content, and compromised structural integrity of the femoral head[9][10]. This process may be closely associated with the mitochondrial apoptotic pathway triggered by mitochondrial dysfunction. The mitochondrial apoptotic pathway is an intrinsic cell death process initiated by mitochondrial damage and dependent on mitochondrial signaling[11]. In SIONFH models, GCs can directly impair mitochondrial function[12], and can also indirectly induce mitochondrial damage by promoting excessive accumulation of reactive oxygen species (ROS)[13][14], which in turn triggers mitochondrial apoptotic pathway and ultimately causing osteoblast apoptosis[11][15]. As an important anti-apoptotic target, the PI3K/AKT signaling pathway has been widely demonstrated to suppress apoptosis by downregulating the expression of pro-apoptotic markers such as Bcl-2-associated X protein (BAX), Bcl-2 homology 3-interacting domain death agonist and cysteine-dependent aspartate-specific proteases[16][17]. In a variety of diseases, including hearing impairment, tumors, and skin lesions, the PI3K/AKT signaling pathway serves as a key regulatory axis of the mitochondrial apoptotic pathway[18][19][20]. However, it remains unclear whether PI3K/AKT signaling participates in GC-induced SIONFH through regulation of the mitochondrial apoptotic pathway.

From the perspective of traditional Chinese medicine theory, SIONFH is mainly caused by kidney deficiency and blood stasis obstruction. In recent years, findings from modern medical and biological studies have also supported this view. Long-term or high-dose use of GCs not only induces nephrotoxicity and aggravates acute kidney injury, but also promotes the recruitment of platelets and erythrocytes, increases the risk of thrombosis, and causes intraosseous microcirculatory disturbance[21][22][23]. In addition, a pathological state of the kidney can disrupt the balance of calcium and phosphate metabolism as well as multiple growth factors, thereby leading to bone loss, which also corroborates the modern biological basis of the traditional Chinese medicine theory that “the kidney governs bone”[24]. As a classical traditional Chinese medicine formula, Guben Zhuyu Decoction (GBZY) exerts effects of strengthening kidney function, removing blood stasis and dredging the collaterals, and reinforcing bone. This is not only highly consistent with the traditional medical understanding of

the pathogenesis of SIONFH, but also corresponds to modern pathological mechanisms induced by GCs, including renal injury, microcirculatory disturbance, and impaired osteogenesis. However, although this formula has shown remarkable efficacy during long-term clinical application, its specific mechanism of action remains unclear. Elucidating the pharmacodynamic material basis and molecular targets underlying its therapeutic effects on SIONFH has therefore become a key issue in current research.

Based on the above background, we proposed the following hypothesis: GBZY may repair glucocorticoid-induced impaired osteogenesis in osteonecrosis of the femoral head by targeting the PI3K/AKT pathway to inhibit the mitochondrial apoptotic pathway. To test this hypothesis, the present study investigated the therapeutic potential of GBZY in reversing suppressed bone formation based on a GC-induced SIONFH model. By integrating HPLC–MS/MS and network pharmacology, we analyzed the key target pathways through which GBZY intervenes in SIONFH, with a particular focus on the mitochondrial apoptotic pathway, thereby providing a basis for its further clinical translation.

2. Materials and Methods

2.1. Animal grouping and treatment

Male Sprague-Dawley rats (6-8 weeks, 200-250 g) were obtained from Changchun Yisi Biotechnology. Animal use was approved by the Animal Ethics Committee of Changchun University of Chinese Medicine (No. 2021355), and all rats were maintained under SPF conditions at 25-30 °C.

Following random allocation, animals entered six parallel cohorts (n = 10 each): vehicle-control (Ctrl), model, GBZY at low (GBZY-L), medium (GBZY-M) or high (GBZY-H) dose, and a positive drug (PD) arm. After 1 week of acclimation, SIONFH was induced *via* combined administration of lipopolysaccharide (LPS; 10 µg/kg, intraperitoneal injection, daily for three successive days) and methylprednisolone (MPS; 40 mg/kg, intramuscular injection, daily for four successive days).

Oral doses of GBZY and PD were calculated according to body surface area. GBZY was administered *via* gavage at 15.43, 30.85, and 61.7 g/kg/day in the GBZY-L, GBZY-M, and GBZY-H groups, respectively. Rats in the PD group received alendronate sodium (Beijing, China; approval No. H20059029) at 6.25 mg/kg/day. Treatments began on the second day after model induction and continued once daily for 4 weeks (2 mL per dose). The Ctrl and model groups were administered equivalent volumes of saline. After treatment, gait and weight-bearing tests were performed. Rats were then euthanized *via* CO₂ inhalation, followed by femoral-head harvesting for downstream analyses.

2.2. GBZY preparation

GBZY was supplied by the Department of Pharmacy, Affiliated Hospital of Changchun University of Chinese Medicine. The detailed composition of GBZY is presented in Table 1. Herbal materials were rinsed with cold water, soaked at a 1:12 (w/v) ratio for 1.5 h, and decocted twice for 2 h each time, with Amomi Fructus and

Citri Reticulatae Pericarpium Viride added during the final 30 min of the first decoction. The filtrates were combined, filtered through a 100-mesh sieve and four layers of 400-mesh gauze, and spun at 3000 rpm for 15 min, and the supernatant fraction was harvested. Extracts were pre-frozen at -80°C for 12 h and then lyophilized for 96 h (Thermo Fisher Scientific, Waltham, MA, USA). The final GBZY powder contained $\leq 2.5\%$ moisture.

Table 1. Herbal medicine composition of GBZY

Chinese herbal medicines	Latin name	Origin	Weight (g)
Lu Jiao Shuang	<i>Cervi Cornu Degelatinatum</i>	Jilin, China	10
Gu Sui Bu	<i>Drynariae Rhizoma</i>	Hubei, China	30
Shu Di Huang	<i>Rehmanniae Radix Praeparata</i>	Henan, China	30
Yin Yang Huo	<i>Epimedii Folium</i>	Shanxi, China	30
Rou Cong Rong	<i>Cistanches Herba</i>	Neimenggu, China	20
Huang Qi	<i>Astragali Radix</i>	Gansu, China	20
Dan Shen	<i>Salvia miltiorrhiza Bunge</i>	Shandong, China	20
San Qi	<i>Notoginseng Radix et Rhizoma</i>	Yunnan, China	20
Yan Hu Suo	<i>Corydalis Rhizoma</i>	Zhe Jiang, China	20
Lu Xian Cao	<i>Pyrolae Herba</i>	Jilin, China	20
Xu Jie	<i>Draconis Sanguis</i>	Indonesia	15
Ru Xiang	<i>Olibanum</i>	Somalia	15
Mo Yao	<i>Myrrha</i>	Somalia	15
Dang Gui	<i>Angelica sinensis Radix</i>	Gansu, China	10
Tu Bie Chong	<i>Eupolyphaga steleophaga</i>	Jiangsu, China	10
Zang Hong Hua	<i>Crocus sativus</i>	Iran	5
Er Cha	<i>Acacia catechu</i>	Yunnan, China	5
Zi Ran Tong	<i>Pyritum</i>	Sichuan, China	5
Sha Ren	<i>Amomi Fructus</i>	Guangdong, China	5
Qin Pi	<i>Citri Reticulatae Pericarpium Viride</i>	Fujian, China	5

2.3. Gait analysis

Gait was analyzed using the DigiGait™ Imaging System (Harvard Apparatus, Holliston, MA, USA) at a treadmill speed of 18 cm/s. The body weight of each rat was recorded prior to testing. Continuous 20-s recordings were captured, and 5-s segments were analyzed to extract parameters including paw area, swing time, and braking time.

2.4. Weight-bearing test

Hindlimb weight-bearing was evaluated using a four-limb weight-bearing apparatus (BIO-DWB2-M, USA). Each rat was allowed to move freely for 5 min, during which a top-mounted camera and base gravity sensors recorded hind paw contact area, supporting force, and stance duration. Data were analyzed using DigiGait™.

2.5. Gross observation

The femoral heads of rats in each group were photographed using a high-definition digital camera. The morphology, color, and surface condition of the articular cartilage of the femoral heads were visually examined and compared among the groups.

2.6. Biomechanical testing

Three-point bending and compression tests were performed on femoral heads using a universal testing machine (INSTRON-2, USA). Maximum load ($F_{\max}$), breaking force (F_{break}), and failure stress (F_{stress}) were recorded to evaluate bone biomechanical properties.

2.7. Micro-computed tomography (CT)

Micro-CT (Quantum GX; PerkinElmer, Waltham, MA, USA) was carried out at 90 kV and 80 μ A for 4 min without interruption. Multi-planar views (coronal, sagittal and transverse) of each femoral head were examined to assess necrotic regions and trabecular porosity. Bone microarchitecture was subsequently extracted with Analyze 12.0, yielding bone mineral density (BMD), structure model index (SMI), bone volume/tissue volume ratio (BV/TV), bone surface/total volume ratio (BS/TV), trabecular thickness (Tb.Th), trabecular spacing (Tb.Sp), and bone surface/bone volume ratio (BS/BV). Using the acquired datasets, we performed three-dimensional reconstruction of the femoral head images.

2.8. Histological staining

Femoral head specimens were fixed in 4% paraformaldehyde, decalcified, paraffin-embedded, and sectioned. These sections were stained with hematoxylin and eosin (H&E) as well as Masson's trichrome. Subsequently, slides were scanned with an M8 slide scanner (Precipoint, Garching, Germany), and staining interfaces were analyzed quantitatively in ImageJ (NIH, Bethesda, MD, USA).

2.9. Immunohistochemistry (IHC)

Paraffin-embedded femoral-head slices were dewaxed, rehydrated, and heat-treated for antigen retrieval. Non-specific sites were blocked with 1% bovine serum albumin. Sections were then probed with primary antibody (4°C, overnight), washed, and incubated with HRP-labeled secondary antibodies. A chromogenic substrate was applied to visualize the target protein, followed by stop solution to terminate the reaction, and slides were mounted. Sections were scanned using a digital slide scanning system, and staining was analyzed and quantified using ImageJ. Detailed information on antibodies is provided in Table 2.

Table 2. Relevant antibody information

Name	Catalog number	Company	Place of origin	Experimental use
COL1A1	14695-1-AP	Proteintech	USA	IHC
ALP	18507-1-AP	Proteintech	USA	IHC/WB
RUNX2	20700-1-AP	Proteintech	USA	IHC/WB
BMP-2	66383-1-Ig	Proteintech	USA	WB

OPN	25715-1-Ap	Proteintech	USA	IHC/WB
OCN	bsm-62874R	Bioss	USA	WB
BCL-2	AF6139	Affinity Biosciences	USA	WB
BAX	F0037	Selleck	USA	WB
PI3K	60225-1-Ig	Proteintech	USA	IHC/WB
P-PI3K	AF3241	Affinity Biosciences	USA	WB
AKT	60203-2-Ig	Proteintech	USA	IHC/WB
P-AKT	66444-1-Ig	Proteintech	USA	WB
β -actin	66009-1-Ig	Proteintech	USA	WB

2.10. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Femoral-head total RNA was extracted with TIANGEN's DP419 kit (Beijing, China) and reverse-transcribed with the All-in-One First-Strand MasterMix (EG15133S, BestEnzymes, Lianyungang, China). qPCR amplification was performed using a real-time PCR system with Taq SYBR® Green qPCR Premix (EG20117M, BestEnzymes). Transcript abundance of runt-related transcription factor 2, bone morphogenetic protein 2 (*BMP-2*), osteopontin (*OPN*), osteocalcin (*OCN*), and alkaline phosphatase (*ALP*) was quantified relative to β -actin via the $2^{-\Delta\Delta CT}$ method. Primer details are provided in Supplementary Tables 1-2.

2.11. Cell culture

MC3T3-E1 subclone 14 cells (CL-0378, Pricella, Wuhan, China) were maintained in MEM- α plus 10% fetal bovine serum (FBS) at 37°C, 5 % CO₂, and the medium was replaced every 48-72 h. At 80–90 % confluence, cells were passaged or cryopreserved.

For induction, logarithmic-phase cells were dissociated, plated at 1.5×10^5 cells/mL, and switched when >70 % confluent to osteogenic medium according to previous studies and preliminary experiments[25]: MEM- α /10 % FBS, 10 nM dexamethasone (D4902, Sigma-Aldrich), 50 μ g/mL ascorbic acid (PHR1008, Sigma-Aldrich) and 10 mM β -glycerophosphate (Y69241, Yuanye, Shanghai, China). Differentiation was monitored daily and evaluated on day 14.

2.12. Cell Counting Kit-8 (CCK-8) assay

Logarithmic-phase cells were enzymatically dissociated, collected via centrifugation, and resuspended in complete culture medium. Cell density was adjusted to 6×10^3 cells per well before seeding into 96-well plates at 100 μ L per well, with five replicates per experimental group. Following incubation periods of 24, 48, or 72 hours,, 100 μ L of CCK-8 working solution (IV08-500, Invigentech, Irvine, CA, USA) was added to each well under light-protected conditions. After a 1.5-hour incubation, absorbance was measured at 450 nm using a microplate reader.

2.13. EdU cell proliferation assay

Cell proliferation was assessed using the BeyoClick™ EdU Cell Proliferation Kit with AF488 (C0071S, Beyotime Biotechnology, Haimen, China). Stained cells were imaged using the EVOS™ M7000 Imaging System (Thermo Fisher Scientific), and fluorescence signals were quantified in ImageJ.

2.14. Mineralized nodule staining

After osteogenic induction, mineralized nodules were stained using an Alizarin Red S staining kit (C0148S, Beyotime Biotechnology). Stained cells were observed and photographed under a light microscope (Nikon, Tokyo, Japan). Stains were dissolved with guanidine hydrochloride solution, and absorbance was measured at 280 nm using a microplate reader for quantitative analysis.

2.15. ALP staining

Following osteogenic induction, a commercial staining kit (C3206, Beyotime Biotechnology) was employed to assess ALP activity. Stained cells were imaged under a light microscope, and analyzed densitometrically with ImageJ.

2.16. Western blotting (WB)

Whole-cell proteins were extracted using RIPA lysis buffer supplemented with PMSF at a 100:1 ratio. After separation via SDS-PAGE, proteins were electrotransferred onto PVDF membranes. Primary antibodies against the target proteins were applied to the membranes and left to bind overnight at 4 °C. Subsequently, the blots were incubated with the corresponding secondary antibodies. Immunoreactive bands were visualized using chemiluminescent substrate, and analyzed densitometrically with ImageJ. Antibody specifications are listed in Table 2.

2.17. Identification of major compounds in GBZY by HPLC-MS/MS

A measured quantity of freeze-dried GBZY powder was placed in a 2-mL polypropylene tube containing one 3-mm steel bead. After adding 1 mL of 70 % methanol, the mixture was homogenized for 3 min at 70 Hz in an automated bead-mill (JXFSTPRP-48), then vortex-mixed for 10 min.. After centrifugation (12 000 × g, 10 min, 4 °C), the supernatant was passed through a 0.22-μm microporous membrane and spiked with an internal standard solution of 2-chlorophenylalanine (100 μg/mL) to a final concentration of 1 mg/L for analysis.

HPLC-MS/MS was performed using an LC-MS system (Vanquish, Q Exactive HF, Thermo Fisher Scientific) on a Zorbax Eclipse C18 column (2.1 × 100 mm, 1.8 μm) maintained at 30 °C. The column was eluted at 0.3 mL min⁻¹ with a gradient of 0.1 % formic acid in water (A) and acetonitrile (B). The injection volume was 2 μL, and the autosampler temperature was maintained at 4°C.

With the heater set to 325 °C, sheath gas to 45 arb, auxiliary gas to 15 arb, sweep gas to 1 arb, spray voltage to 3.5 kV, capillary temperature to 330 °C and S-Lens RF level to 55 %, the MS measurements were executed in both positive- and negative-ion electrospray modes.. Data were acquired in full scan (m/z 100–1500) and complemented by data-dependent MS/MS conditions (TopN = 5) with a resolution of

120,000 (MS¹) and 60,000 (MS²). High-energy collision dissociation was used for fragmentation. Compound identification was performed using Compound Discoverer 3.3, with retention time correction, peak detection, and extraction. MS/MS spectra were matched against the mzCloud online database (Thermo Fisher Scientific), mzVault local database (Thermo Fisher Scientific), and published literature to ensure accurate chemical identification.

2.18. Network pharmacology analysis and molecular docking

Potential SIONFH-related targets were identified according to the HPLC-MS/MS results. “Steroid-induced osteonecrosis of the femoral head” was used as the keyword to systematically search the GeneCards, OMIM, PharmGkb, DrugBank, and TTD (<https://db.idrblab.net/ttd/>) databases. All targets from the five databases were merged in Excel (Microsoft, Redmond, WA, USA), duplicates were removed, and targets were standardized using UniProt. Overlapping targets between GBZY compounds and SIONFH were determined using R software (<https://www.r-project.org/>), and a Venn diagram was generated. A “drug–compound–target” network was constructed using Cytoscape 3.9.2.

To explore interactions among core targets, the shared subset was uploaded to STRING (Homo sapiens, confidence ≥ 0.7). The exported TSV file was imported into Cytoscape 3.9.2 to generate a protein–protein interaction (PPI) network in which node dimensions and color reflect degree values, whereas edge thickness mirrors combined interaction scores. Topological filtering—degree, local average connectivity, eigenvector centrality, network centrality, and betweenness centrality—was applied to prioritize hub targets.

Functional annotation was performed using DAVID (<https://david.ncifcrf.gov/summary.jsp>). Gene Ontology (GO) terms spanning biological process (BP), cellular component (CC), and molecular function (MF) were retrieved, together with Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways relevant to GBZY activity against SIONFH. The ten most significant GO categories and twenty top-ranking KEGG routes ($P < 0.05$) were plotted to infer putative mechanisms. Molecular docking corroborated binding compatibility between GBZY bioactives and the core proteins. Ligands and receptor structures were prepared in AutoDockTools 1.5.6 (<https://ccsb.scripps.edu/mgltools/>), converted to PDBQT format, and docked with AutoDock 4 (<https://autodock.scripps.edu/>). The four best docking results exhibiting the lowest binding energies were visualized in PyMOL (<https://www.pymol.org/>).

2.19. ROS analysis

Suspended cells were collected by centrifugation and labeled with H2DCFDA reagent (HY-D0940, MedChemExpress, Monmouth Junction, NJ, USA) to detect intracellular ROS levels. Fluorescence intensity was quantified using a flow cytometer (Beckman Coulter, Brea, CA, USA).

2.20. Mitochondrial membrane potential (MMP) assay

MMP was measured with a JC-1 assay kit (C2006, Beyotime Biotechnology). Fluorescent images were captured using the EVOS™ M7000 Imaging System, and staining was analyzed and quantified with ImageJ.

2.21. Mitochondrial fluorescence staining

Mitochondria were labeled using the MitoTracker Deep Red 633 kit (C1997S, Beyotime Biotechnology). Fluorescence images were acquired with the EVOS™ M7000 Imaging System, and staining was analyzed and quantified using ImageJ.

2.22. Statistical analysis

All data were first entered into Excel. Statistical processing was conducted in GraphPad Prism 9.5 (GraphPad, Boston, MA, USA). Categorical data were analyzed using the chi-squared test, whereas continuous data were analyzed using one-way ANOVA. When data did not satisfy a normal distribution, a rank-sum test was applied. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. GBZY improves behavioral, mechanical, and imaging phenotypes in rats with SIONFH

In this study, a rat model of SIONFH was induced by combined administration of LPS and MPS to evaluate the therapeutic potential of GBZY in alleviating SIONFH in rats. Following GC induction, gait analysis revealed significant behavioral impairments in the model group compared with the findings in the Ctrl group, including increased swing and braking times, decreased stride length, reduced gait symmetry, and increased forelimb weight support (all $P < 0.05$). Treatment with GBZY and PD partially reversed these deficits. Rats in the GBZY and PD groups displayed improvements in swing time, braking time, stride length, and gait symmetry, along with reduced forelimb weight support, with the greatest behavioral improvement recorded in the GBZY-H group ($P < 0.01$, Fig. 1A).

After model induction, weight-bearing tests illustrated that compared with the findings in Ctrl rats, model rats showed a significant reduction in the maximum hindlimb bearing force (rear left/right paw weight), maximum bearing area (rear left/right paw area), and maximum bearing duration (rear left/right posed duration; all $P < 0.001$), indicating that the combined administration of LPS and MPS successfully induced SIONFH with impaired weight-bearing capacity. Both GBZY and PD significantly increased the maximum bearing force, bearing area, and bearing duration of the hind limbs in rats with SIONFH. Notably, GBZY-H exerted marked therapeutic effects on the LPS+MPS-induced decreases in weight-bearing ability, duration, and area (all $P < 0.001$, Fig. 1B).

After euthanasia, macroscopic examination of the femoral heads revealed substantial morphological alterations in the model group, including cartilage exfoliation, dull coloration, and localized bone fragility. These pathological changes were notably alleviated in the GBZY and PD groups, which displayed improved cartilage integrity, bone texture, and color (Fig. 1C). To further evaluate the

biomechanical integrity of the femoral heads, three-point bending and compression tests were performed. Compared with the findings in the Ctrl group, the model group exhibited significant reductions in $F_{\max}$, F_{break} , and F_{stress} (all $P < 0.001$), indicating compromised cortical bone strength and mechanical stability after model induction. GBZY and PD treatment significantly improved these biomechanical indices, thereby enhancing the load-bearing capacity and strength of cortical bone in rats with SIONFH (all $P < 0.001$, Fig. 1D).

Micro-CT and three-dimensional reconstruction of the femoral head indicated that after treatment, partial imaging manifestations of SIONFH remained in the model, GBZY-L, and PD groups, whereas the necrotic condition was significantly improved in the GBZY-M and GBZY-H groups (Fig. 1E). Meanwhile, both GBZY and PD increased BMD, Tb.Th, BV/TV, BS/BV, and BS/TV and significantly reduced Tb.Sp and SMI in rats with SIONFH. In particular, the overall therapeutic effect was strongest in the GBZY-H group ($P < 0.001$, Fig. 1E).

3.2. GBZY attenuates histopathological injury and restores expression of osteogenic differentiation markers in rats with SIONFH

To further assess the reparative capacity of GBZY on bone-forming capacity in rats with SIONFH, the present study conducted analyses from two aspects: histopathological observation and detection of osteogenic differentiation markers. H&E staining showed that, compared with the Ctrl group, the model group exhibited a significant reduction in trabeculae, disappearance of osteocyte nuclei, empty bone lacunae, ruptured adipocytes, and infiltration of inflammatory cells in the surrounding area. Conversely, varying degrees of recovery were observed in the femoral heads of rats in the GBZY and PD groups, which were characterized by an increased number of trabeculae with a more orderly arrangement and the restoration of nuclei in osteoblasts (Fig. 2A). Masson's staining illustrated a reduction in the nuclear staining of hematogenous bone marrow cells in the necrotic area and a large increase in the area of fibrotic scar tissue in the model group versus the findings in the Ctrl group. The SIONFH lesions both affected the femoral head and extended to the femoral neck. The fibrous lesions in the femoral heads of rats in the GBZY-M, GBZY-H, and PD groups were significantly improved (Fig. 2B). To further evaluate the condition of the femoral heads in rats with SIONFH, ImageJ was used to quantitatively analyze the percentage of empty lacunae in HE-stained samples and the proportion of mature bone tissue in Masson-stained samples in each group. The empty-lacuna ratio was markedly higher in the model group than in the Ctrl group ($P < 0.001$), whereas treatment with GBZY and PD reversed this trend (both $P < 0.001$). In Masson's staining, obvious fibrotic damage in bone tissue was noted in the model group ($P < 0.001$), whereas the area of mature bone tissue was significantly increased in the GBZY and PD groups (all $P < 0.001$). These results suggest that GBZY can significantly delay and inhibit bone mass loss in rats with SIONFH (Fig. 2A–B).

Finally, mRNA illustrated that the osteogenic markers *OPN*, *OCN*, *RUNX2*, *BMP-2*, and *ALP* were significantly downregulated in the model group (all $P < 0.001$), whereas GBZY at different doses significantly promoted their expression (all $P <$

0.05). IHC confirmed that ALP, RUNX2, BMP-2, and collagen type I alpha 1 (COL1A1) protein expression was enhanced in GBZY-treated rats (all $P < 0.05$, Fig. 2C–D).

3.3. GBZY promotes the proliferation and osteogenic differentiation of DEX-induced MC3T3-E1 cells

To further investigate the in vitro mechanism by which GBZY promotes osteogenesis in SIONFH, MC3T3-E1 cells induced with DEX were treated with different concentrations of GBZY. CCK-8 assays performed at 24, 48, and 72 h showed that GBZY promoted osteoblast proliferation. Concentrations of 1, 2, and 4 $\mu\text{g/mL}$ were therefore selected as the low-, medium-, and high-dose groups, respectively, for subsequent experiments (all $P < 0.05$, Fig. 3A).

Osteogenic differentiation of osteoblasts is an important pathway for maintaining bone strength, and osteogenic proteins and mineralization are key indicators of osteogenic differentiation[26]. A DEX-induced osteogenic inhibition model was constructed in MC3T3-E1 cells, followed by intervention with low, medium, and high concentrations of GBZY. EdU staining demonstrated that GBZY enhanced the proliferative ability of DEX-treated MC3T3-E1 cells, with the greatest effect observed for the highest concentration ($P < 0.001$, Fig. 3B). DEX inhibited ALP expression and mineralization in MC3T3-E1 cells. GBZY treatment reversed this inhibition, increasing ALP content and enhancing mineralization (both $P < 0.001$, Fig. 3C).

Further evaluation of osteogenic marker gene expression revealed that GBZY at concentrations of 1, 2, and 4 $\mu\text{g/mL}$ upregulated osteogenic-related genes, including *OPN*, *ALP*, *RUNX2*, *OCN*, and *BMP-2*, in DEX-exposed MC3T3-E1 cells (all $P < 0.05$, Fig. 3D). This effect was further confirmed at the protein level, with GBZY enhancing the expression of *OPN*, *ALP*, *RUNX2*, *OCN*, and *BMP-2* (all $P < 0.05$, Fig. 3E). These results indicate that GBZY can promote cell proliferation and enhance the mineralization and osteogenic capacity of MC3T3-E1 cells.

3.4 HPLC-MS/MS and network pharmacology analysis of GBZY

The major chemical components of GBZY were analyzed using HPLC-MS/MS. Based on relative abundance, each compound was identified according to previous studies. In total, 103 compounds were identified under positive-ion mode, and 65 compounds were identified under negative-ion mode. Detailed information on the chemical components is provided in Supplementary Tables 3–4. The top five compounds identified in each ion mode are highlighted in Fig. 4A.

Based on the MS results, network pharmacology was employed to predict potential targets and pathways of GBZY in SIONFH. A Venn diagram of drug–disease targets revealed 88 overlapping targets between GBZY and SIONFH (Fig. 4B). These targets were imported into Cytoscape 3.9.2 to construct a drug–component–target network, revealing the importance of individual compounds. Among them, tanshinone IIA, kaempferol, quercetin, β -sitosterol, and luteolin displayed higher significance, suggesting their key roles in treating SIONFH (Fig. 4B).

To further explore the pharmacological mechanisms of GBZY in SIONFH, a PPI

network was generated using STRING. Network topology analysis identified hub nodes based on degree, local average connectivity, eigenvector centrality, betweenness centrality, and network centrality. AKT1, IL-6, estrogen receptor 1 (ESR1), and tumor necrosis factor (TNF) were identified as four core targets (Fig. 4C).

These four key targets were subjected to GO and KEGG enrichment analyses using the DAVID database. GO enrichment yielded 2114 highly enriched genes at a threshold of $P < 0.01$, including 1521 BP-related genes, 28 CC-related genes, and 94 MF-related genes. Among the top 10 GO terms, GBZY was implicated in regulating transcription, gene expression, signal transduction, inflammatory responses, cell proliferation, and apoptosis inhibition (Fig. 4D). KEGG profiling identified 20 significantly enriched pathways ($P < 0.01$). Among them phosphoinositide 3-kinase (PI3K)/serine/threonine kinase B (AKT) displaying the greatest enrichment, suggesting that GBZY exerts its therapeutic effect on SIONFH *via* this pathway (Fig. 4D).

Based on the drug–component–target and PPI networks, molecular docking was performed among five key compounds (β -sitosterol, luteolin, quercetin, kaempferol, and tanshinone IIA) and four core targets (AKT1, ESR1, TNF, and IL-6). Based on binding energies lower than -5 kcal/mol, successful docking was noted between AKT1 and quercetin (-9 kcal/mol), IL-6 and luteolin (-8.1 kcal/mol), TNF and tanshinone IIA (-7.7 kcal/mol), and ESR1 and β -sitosterol (-8.3 kcal/mol). These results suggest that AKT1 is the key target of GBZY in the PI3K/AKT signaling pathway for treating SIONFH (Fig. 5A).

Consistent with the KEGG enrichment and molecular docking results, IHC and WB demonstrated that GBZY increased PI3K and AKT protein expression (both $P < 0.001$) and the ratios of p-PI3K/PI3K and p-AKT/AKT (both $P < 0.05$), indicating enhanced PI3K/AKT signaling and phosphorylation (Fig. 5B–C).

3.5. GBZY inhibits the mitochondrial apoptotic pathway and protects DEX-induced MC3T3-E1 cells from apoptosis

Osteoblast apoptosis is a critical event in the dynamic balance of bone metabolism. The PI3K/AKT signaling pathway is an important anti-apoptotic pathway and also serves as a key regulatory axis of mitochondrial function[27][28]. Therefore, this study further explored the effects of GBZY on the mitochondrial apoptotic pathway in MC3T3-E1 cells. ROS are important triggers of apoptosis, and the flow cytometry results demonstrated that GBZY concentration-dependently reduced DEX-induced ROS levels ($P < 0.001$, Fig. 6A) and cell apoptosis rates ($P < 0.001$, Fig. 6B). At the protein level, GBZY inhibited the expression of the pro-apoptotic factor Bcl-2-associated X protein (BAX) while promoting the expression of the anti-apoptotic factor (BCL-2; both $P < 0.05$, Fig. 6C).

Mitochondrial function is a crucial regulator of apoptosis, and changes in MMP are closely associated with apoptotic progression[29]. JC-1 staining revealed a significant reduction in MMP in DEX-induced MC3T3-E1 cells, as indicated by a marked increase in green fluorescence ($P < 0.001$). GBZY treatment significantly

restored MMP, as indicated by a decrease in green fluorescence ($P < 0.01$, Fig. 6D). Furthermore, a novel fluorescent probe (MitoTracker Deep Red 633), which specifically stains mitochondria in live cells, was used to evaluate MMP. DEX induction markedly reduced red fluorescence in the model group ($P < 0.001$), indicating a significant decline in MMP. GBZY treatment restored MMP in MC3T3-E1 cells, as evidenced by increased red fluorescence ($P < 0.05$, Fig. 6E).

3.6. GBZY attenuates apoptosis in DEX-stimulated MC3T3-E1 cells by activating the PI3K/AKT signaling pathway and inhibiting the mitochondrial apoptotic pathway

To further verify that GBZY inhibits the mitochondrial apoptotic pathway in osteoblasts through the PI3K/AKT signaling pathway, we conducted more in-depth investigations using the PI3K inhibitor LY294002 (Cat. No. S1105), which was purchased from Selleck Chemicals. Treatment with 10 μ M LY294002 reduced the expression of PI3K/AKT pathway-related proteins activated by GBZY and inhibited their phosphorylation ($P < 0.01$, Fig. 7A). We found that LY294002 attenuated the therapeutic effects of GBZY by inhibiting the activation of the PI3K/AKT signaling pathway, resulting in increased ROS accumulation and apoptosis (both $P < 0.01$, Fig. 7B–C). At the protein level, GBZY inhibited BAX expression and promoted BCL-2 expression, and these anti-apoptotic effects were reversed by LY294002 (all $P < 0.05$, Fig. 7D).

We further explored whether LY294002 could counteract the effect of GBZY on mitochondrial function. JC-1 and MitoTracker Deep Red staining revealed that GBZY increased MMP in DEX-induced osteoblasts, whereas the addition of LY294002 reversed this effect ($P < 0.05$, Fig. 7E–F).

4. Discussion

SIONFH is a highly prevalent and disabling disorder in which prolonged glucocorticoid therapy leads to osteonecrosis of the femoral head, with an incidence of approximately 9% – 40%[30][31]. GCs can cause the progressive accumulation of osteoblast apoptosis in the femoral head through the mitochondrial apoptotic pathway, thereby disrupting the mechanical function of the osteocyte-lacunar-canalicular system, reducing bone formation, and ultimately triggering a series of irreversible pathological events characterized by femoral head collapse[32]–[35]. However, antiresorptive agents such as alendronate sodium and zoledronic acid remain the first-line drugs for delaying femoral head collapse in SIONFH, and effective alternative or adjunctive therapies capable of promoting bone formation are still lacking[7][8].

As an alternative or adjunctive therapeutic strategy, traditional Chinese medicine has demonstrated considerable therapeutic potential in various skeletal disorders[36][37]. A meta-analysis further showed that traditional Chinese medicine can effectively improve pain, Harris Hip Score, and radiographic changes in ONFH[38]. As a natural medicinal formula, GBZY has shown favorable clinical efficacy in the treatment of SIONFH and holds promise as an alternative or adjunctive therapy, although its specific mechanism remains unclear. In the present study, we

systematically evaluated the reparative effects of GBZY on osteogenesis in SIONFH through both animal and cell experiments. By integrating HPLC-MS/MS and network pharmacology analyses, we screened candidate active constituents and signaling cascades through which GBZY intervenes in SIONFH, and further experimentally validated its potential mechanism.

Both short-term and long-term high-dose GC administration can cause microfractures of the trabeculae within the femoral head, which subsequently lead to collapse[39]. Trabeculae play a critical role in maintaining skeletal architecture. Once the trabecular structure within the femoral head becomes disorganized, the femoral head undergoes structural deterioration, leading to reduced mechanical properties, localized stress concentration, and increased contact stress, thereby promoting the occurrence of subchondral fractures and femoral head collapse[40]. Our findings in the present study further confirmed this phenomenon. Rats in the model group exhibited marked behavioral impairment, significantly reduced hindlimb weight-bearing, and femoral heads characterized by cartilage shedding, a dull appearance, bone fragility, and decreased biomechanical properties (Fig. 1A-D). Further analyses revealed that GCs induced substantial alterations in trabecular structural parameters of the femoral head, including BMD, BV/TV, BS/BV, BS/TV, Tb.Sp, Tb.Th, and SMI (Fig. 1E), accompanied by pathological features indicative of femoral head necrosis, such as disappearance of osteocyte nuclei, empty lacunae, inflammatory cell infiltration in the surrounding tissue, and an increased empty lacuna rate (Fig. 2A-B).

The structural integrity of trabecular bone depends on the effective differentiation and function of osteoblasts. Among these, the activity of key osteogenic differentiation markers, including COL1A1, ALP, RUNX2, OPN, OCN, and BMP-2, represents a central manifestation of bone-forming capacity[41]. Through multiple approaches, including behavioral assessment, imaging, and histopathological analysis, we found that GBZY reversed the abovementioned behavioral impairments and pathological changes (Fig. 1; Fig. 2A-B), increased the gene and protein expression of osteogenic differentiation markers, including ALP, RUNX2, OPN, OCN, BMP-2, and COL1A1 (Fig. 2C-D), and enhanced bone-forming capacity in rats with SIONFH. *In vitro*, MC3T3-E1 cells were used as a primary model for osteogenesis research owing to their osteoblast-like properties of human osteoblasts[42]. GBZY promoted MC3T3-E1 proliferation, osteogenic commitment, and matrix mineralization and increased ALP, RUNX2, OPN, OCN, and BMP-2 expression, thereby improving SIONFH-associated bone remodeling defects (Fig. 3). These results confirm that GBZY improves SIONFH symptoms and pathological features by enhancing osteogenic gene and protein expression.

The mechanism of bone remodeling is complex, and it is essential to identify the key targets and pathways through which GBZY promotes bone remodeling. Therefore, we analyzed and characterized the major chemical components of GBZY using HPLC-MS/MS, identifying more than 100 compounds (Supplementary Tables 3-4). Subsequently, we used network pharmacology to build an interaction network linking these compounds with disease-related targets of SIONFH, revealing 88 overlapping

618 targets. Among them, tanshinone IIA, kaempferol, quercetin, β -sitosterol, and luteolin
619 were identified as five key compounds for the treatment of SIONFH. These five
620 compounds play significant regulatory roles in bone remodeling. Tanshinone IIA can
621 enhance ALP and RUNX2 expression in bone marrow-derived mesenchymal stem
622 cells, thereby promoting osteogenic differentiation[43][44]. Kaempferol can increase
623 the expression of osteogenic factors such as osterix and RUNX2, thereby alleviating
624 DEX-induced apoptosis in MC3T3-E1 cells[45]. Similarly, quercetin modulates the
625 metabolic balance of mesenchymal stem cells, osteoblasts, and osteoclasts through
626 anti-inflammatory and antioxidant pathways, thereby promoting bone remodeling[46].
627 β -sitosterol and luteolin exert unique effects in bone remodeling by suppressing
628 adipogenic differentiation and regulating the dynamic balance between adipogenesis
629 and osteogenesis[47][48]. To further elucidate the pharmacological mechanisms of
630 GBZY in treating SIONFH, topological analysis, GO enrichment analysis, and KEGG
631 pathway annotation were performed(Fig. 4). The results indicated that GBZY action
632 in SIONFH is closely associated with positive regulation of transcription,
633 upregulation of gene expression, promotion of cell proliferation, and inhibition of
634 apoptosis. The PI3K/AKT signaling pathway was identified as a key pathway
635 mediating GBZY's therapeutic effects, a finding that was further validated by
636 molecular docking analysis, IHC, and WB (Fig. 5B-C).

637 The PI3K/AKT pathway is one of the core signaling pathways regulating the
638 mitochondrial apoptotic pathway. In SIONFH, GCs can promote the activation of the
639 transcription factor FOXO1 by inhibiting the PI3K/AKT signaling pathway, thereby
640 upregulating the expression of apoptotic proteins such as BAX and caspase-3 and
641 ultimately inducing osteoblast apoptosis[49]. Conversely, activation of PI3K/AKT can
642 modulate the mitochondrial apoptotic pathway, downregulate the expression of
643 caspase-3, caspase-9, BAX, and cytochrome c, and enhance the expression of the
644 anti-apoptotic protein BCL-2, thereby attenuating DEX-induced osteoblast
645 apoptosis[50]. Among these factors, BCL-2 and BAX are the central regulators of the
646 mitochondrial apoptotic pathway[51][52]. Based on the above mechanisms, the
647 present study further investigated whether GBZY could ameliorate the progression of
648 the mitochondrial apoptotic pathway in DEX-induced MC3T3-E1 cells. The results
649 demonstrated that GBZY concentration-dependently reduced DEX-induced ROS
650 generation, downregulated BAX, upregulated BCL-2, and effectively suppressed cell
651 apoptosis (Fig. 6A-C). In addition, DEX treatment significantly decreased MMP in
652 MC3T3-E1 cells, whereas GBZY treatment markedly reversed this effect, indicating
653 that GBZY effectively improved mitochondrial function (Fig. 6D-E). To clarify the
654 critical role of the PI3K/AKT pathway in GBZY's mechanism of action, the
655 PI3K-specific inhibitor LY294002 was employed. The results indicated that
656 LY294002 effectively reversed GBZY-induced activation of the PI3K/AKT pathway
657 and attenuated the protective effects of GBZY on ROS reduction, apoptosis inhibition,
658 BAX/BCL-2 regulation, and MMP restoration (Fig. 7A-F).

659 Taken together, the above findings indicate that activation of the mitochondrial
660 apoptotic pathway resulting from mitochondrial dysfunction is a key mechanism
661 underlying impaired osteogenesis in SIONFH. GBZY promotes the osteogenic

capacity of SIONFH by activating the PI3K/AKT signaling pathway, and its mechanism of action is mediated through inhibition of the mitochondrial apoptotic pathway. In MC3T3-E1 cells treated with LY294002, suppression of the activated PI3K/AKT signaling pathway further confirmed an essential role of this pathway in the inhibitory effect of GBZY on the mitochondrial apoptotic pathway. However, considering the multi-component nature in traditional Chinese medicine formulas, the intricate in vivo effects of the multiple constituents of GBZY, as well as their deeper influences on mitochondrial dynamics, such as morphology and biogenesis, still warrant further investigation. Detailed pharmacokinetic studies of the identified active components are essential, and elucidating the mechanisms of the core active constituents of GBZY, optimizing component combinations, and enhancing therapeutic efficacy will constitute important directions for future research.

5. Conclusion

This study demonstrates that GBZY promotes osteogenesis in SIONFH by activating the PI3K/AKT signaling pathway and thereby inhibiting the mitochondrial apoptotic pathway. Among these effects, suppression of the mitochondrial apoptotic pathway in osteoblasts represents a key mechanism underlying the therapeutic action of GBZY against SIONFH, which depends on activation of the PI3K/AKT signaling pathway (Fig. 8). This causal relationship was ultimately verified by experiments using the inhibitor LY294002: when the PI3K/AKT pathway was inhibited, the abovementioned protective effects of GBZY were completely reversed, supporting a central role of the PI3K/AKT pathway in the mechanism of action of GBZY.

Abbreviations

SIONFH	Steroid-induced osteonecrosis of the femoral head
GC	Glucocorticoid
DEX	Dexamethasone
BMD	Bone mineral density
BV/TV	Bone volume/tissue volume ratio
BS/BV	Bone surface/bone volume ratio
BS/TV	Bone surface/total volume ratio
Tb.Sp	Trabecular spacing
Tb.Th	Trabecular thickness
SMI	Structure model index
HE	Hematoxylin and Eosin
IHC	Immunohistochemistry
RT-qPCR	Quantitative real-time reverse transcription polymerase chain reaction
ALP	Alkaline phosphatase
WB	Western blotting
BMP-2	Bone morphogenetic protein 2
OPN	Osteopontin
OCN	Osteocalcin

BCL-2	B cell lymphoma/leukemia-2
BAX	Bcl-2-associated X protein
COL1A1	Collagen type I alpha 1
PI3K	Phosphoinositide 3-kinase
AKT	Serine/threonine kinase B
ROS	Reactive oxygen species

Author contributions

Guochan Wang: Writing – original draft, cell experiments, data curation, data analysis. **Yan Zhong:** Animal experiments, data curation, data analysis. **Yu Bai:** Investigation, data curation, data analysis. **Guoyong Zhang:** Investigation, data curation, data analysis. **Chengan Yang:** Network pharmacology research, data curation, data analysis. **Yuliang Cai:** Data curation, data analysis. **Yi Qu:** Investigation, data management. **Xinghua Jiang:** Investigation, data management. **Xudong Zhang:** Investigation, data management. **Wenjun Cai:** Investigation, data management, funding acquisition. **Haisi Dong:** Review & editing. **Wenhai Zhao:** Review & editing. **Changwei Zhao:** Writing – review & editing, funding acquisition.

Ethics Approval and Consent to Participate

The animal experiments were approved by the Animal Ethics Committee of Changchun University of Chinese Medicine (Approval No.: 2021355).

Consent for Publication

We declare that the publisher has obtained authorization from the authors to publish the related research findings.

Declaration of Competing Interest

We declare that there are no competing interests with any individual or institution.

Acknowledgement

We sincerely appreciate the anonymous reviewers for their valuable, perceptive comments. Also, gratitude goes to Editage (www.editage.cn) for its meticulous technical editing of the manuscript.

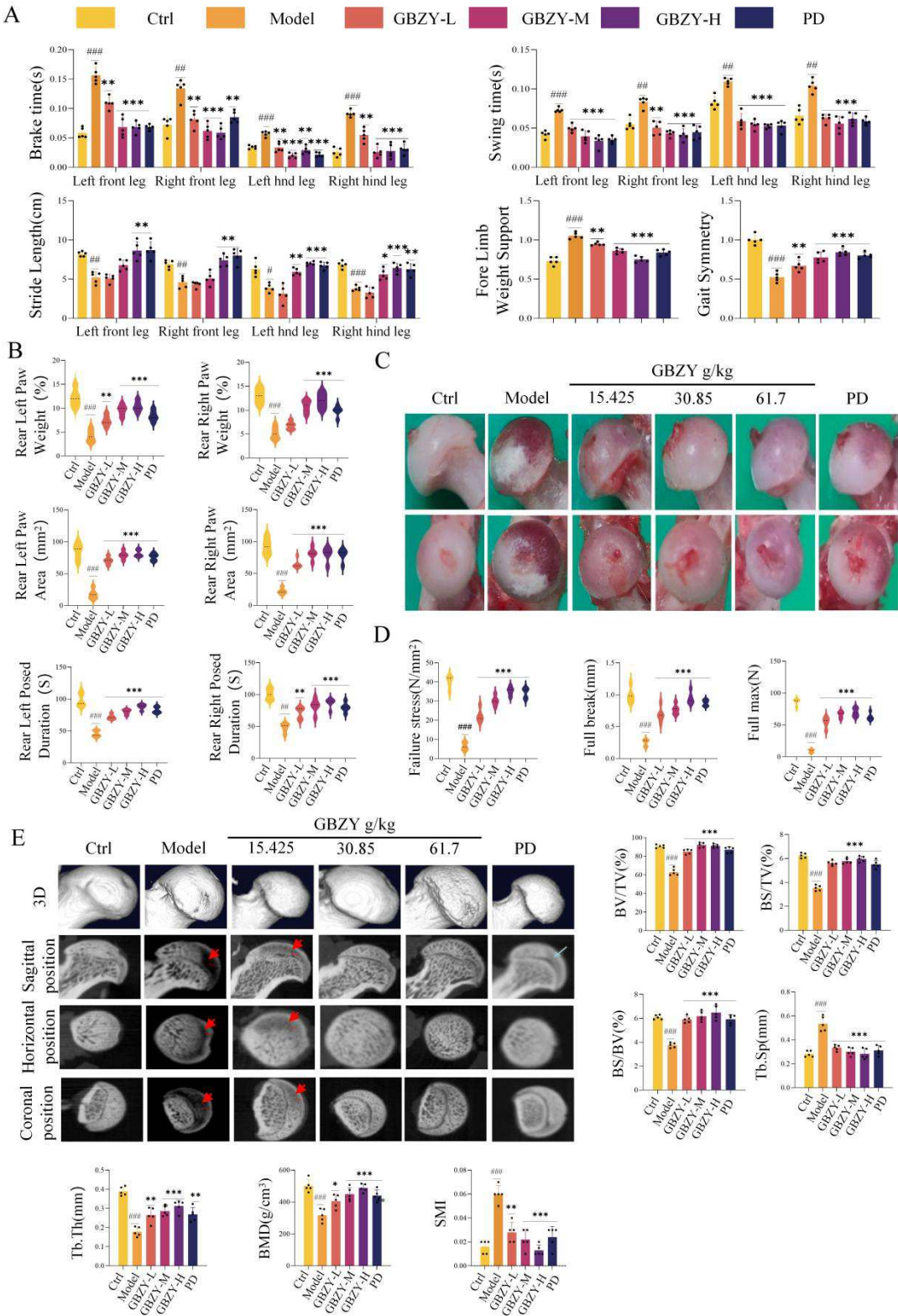
Funding

This study was supported by the Jilin Province Science and Technology Development Plan Project (No. YDZJ202201ZYTS216) and the Jilin Province Department of Education Science and Technology Research Project (No. JJKH20250673BS).

Data Availability

All necessary data are included in the article. For additional data, please contact the corresponding author.

750
751



752

753 Fig. 1. GBZY improves behavior and imaging outcomes in rats with SIONFH.

754 A. Experimental workflow and detection scheme. B. Representative gait analysis of rats (n = 5), including swing

755 time, braking time, stride length, gait symmetry, and forelimb support. C. Weight-bearing test results of rats (n = 5),

including maximum hindlimb bearing force, maximum bearing area, and maximum bearing duration. D. Gross morphology of rat femoral heads. E. Three-point bending and compression test results ($n = 5$), including F_{max} , F_{break} , and F_{stress} . F. Micro-CT and 3D reconstruction results ($n = 5$), including BV/TV, BS/TV, BS/BV, Tb.Sp, Tb.Th, BMD, and SMI. Ctrl vs. model, $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$; model vs. GBZY and PD, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

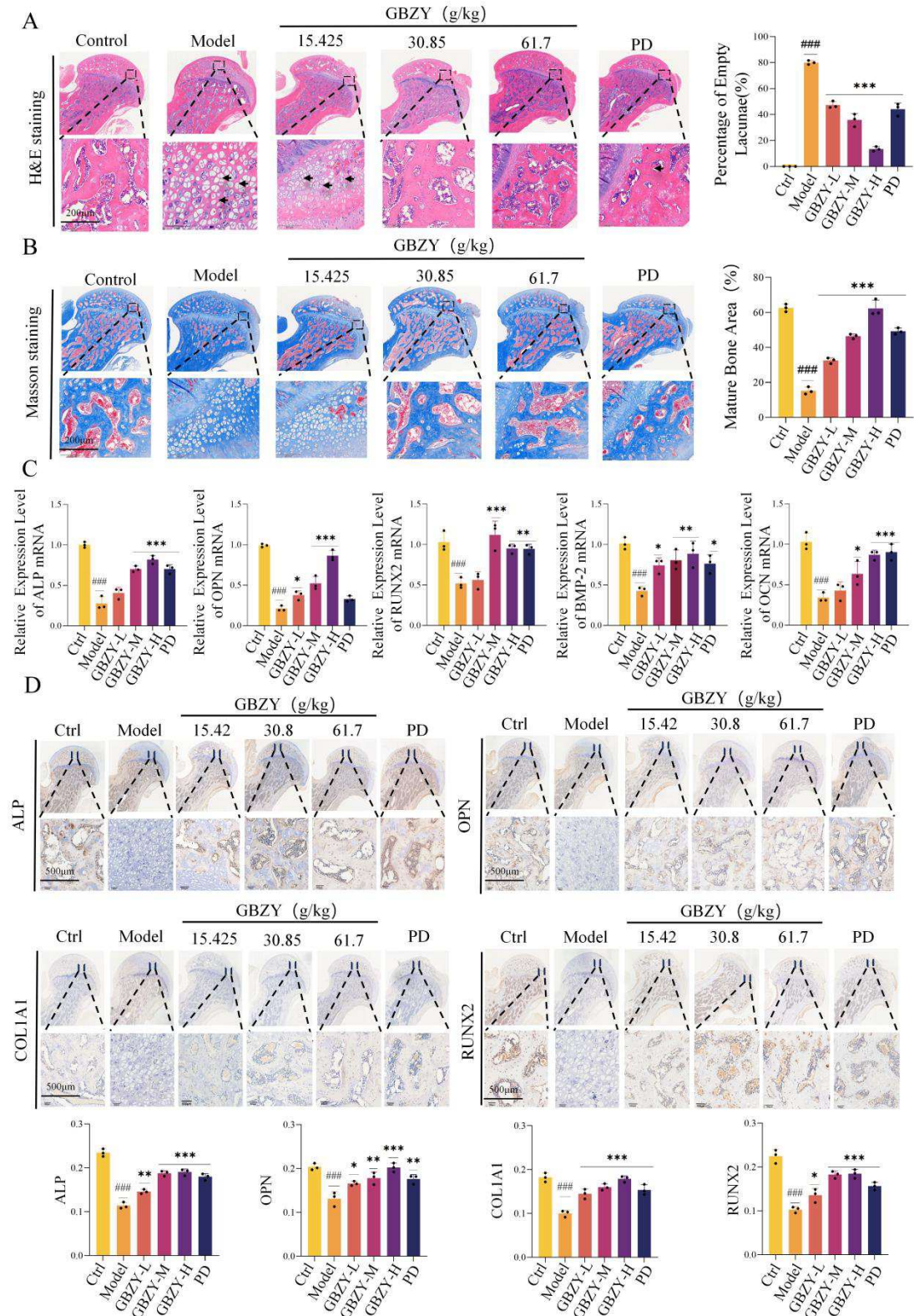


Fig. 2. GBZY enhances osteogenic capacity in rats with SIONFH. A. HE staining results (n = 3) (200μm). B. Masson's staining results (n = 3) (200μm). C. RT-qPCR analysis of osteogenic markers (n = 3), including *ALP*, *OPN*, *RUNX2*, *BMP-2*, and *OCN*. D. IHC of osteogenic markers (n = 3) (500μm), including *ALP*, *OPN*, *COL1A1*, and *RUNX2*. Ctrl vs. model, #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001; model vs. GBZY and PD, **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

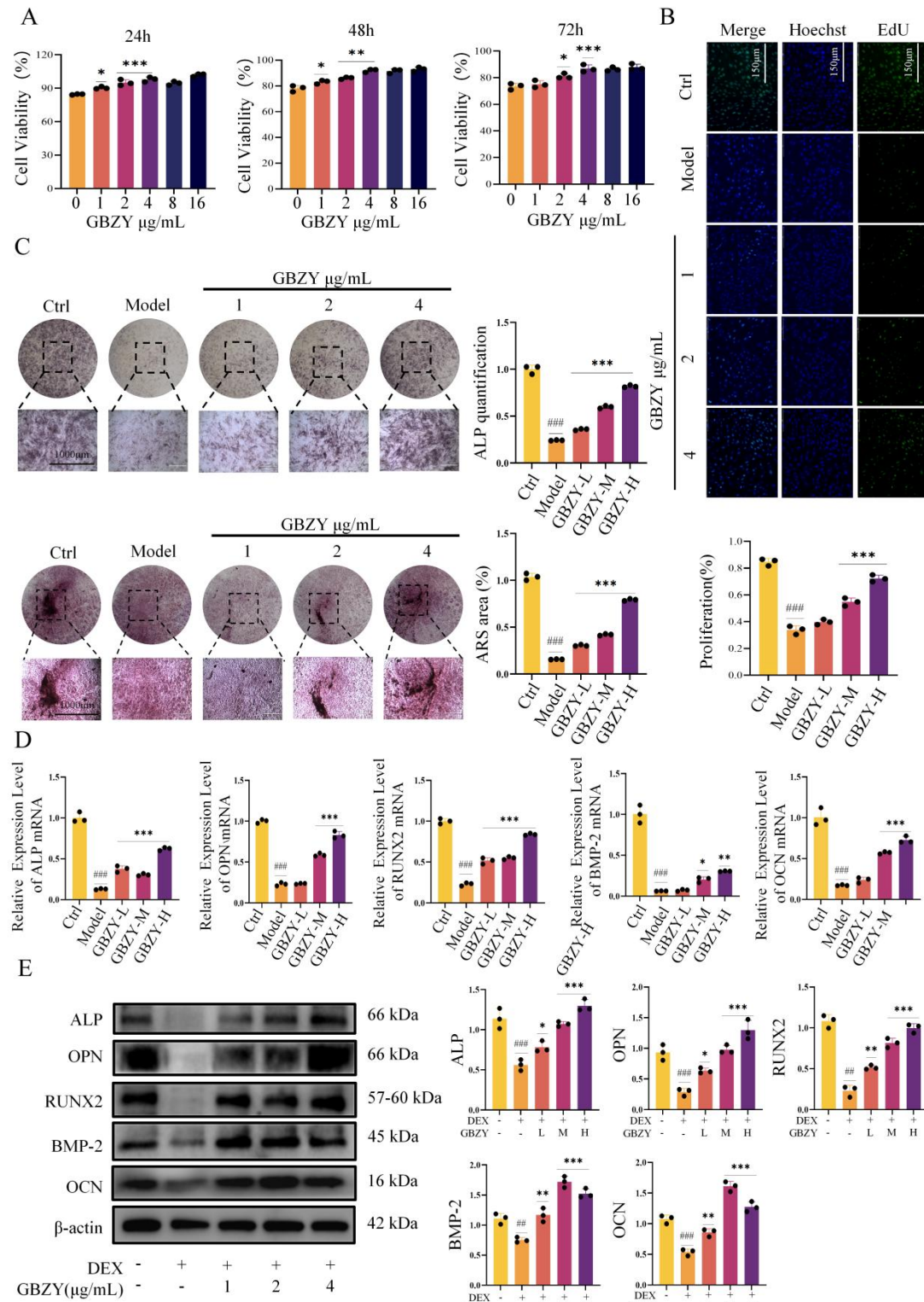


Fig. 3. GBZY improves the proliferation and osteogenic differentiation of DEX-induced MC3T3-E1 cells. A. CCK-8 assay results (n = 3). B. EdU staining results (n = 3) (150μm). C. ALP staining and mineralized nodule staining results (n = 3) (1000μm). D. RT-qPCR analysis of osteogenic markers (n = 3), including *ALP*, *BMP-2*, *RUNX2*, *OCN*, and *OPN*. E. WB of osteogenic markers (n = 3), including ALP, BMP-2, RUNX2, OCN, and OPN. Ctrl vs. model, #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001; model vs. GBZY, **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

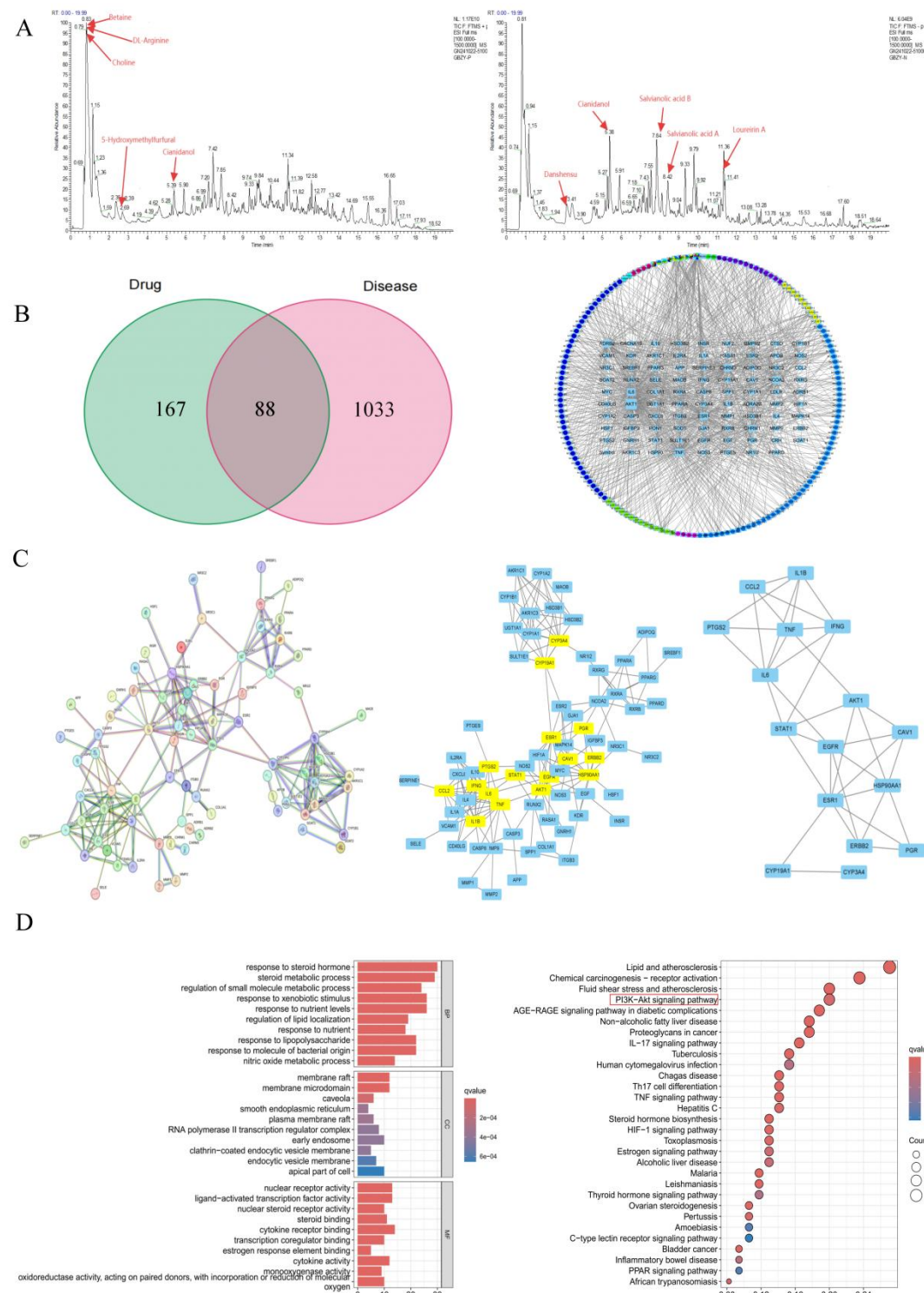


Fig. 4. MS and bioinformatic analysis of GBZY. A. GBZY ion chromatograms. Chemical components with the top five relative contents are highlighted in red for both the positive ion mode (betaine, choline, DL-arginine, cyanidanol, and 5-hydroxymethylfurfural) and negative ion mode (salvianolic acid B, cyanidanol, loureirin A, salvianolic acid A, and danshensu B). B. Venn diagram of drug–disease target intersections and the drug–component–target network. C. PPI network of intersecting genes. D. Functional enrichment analysis.

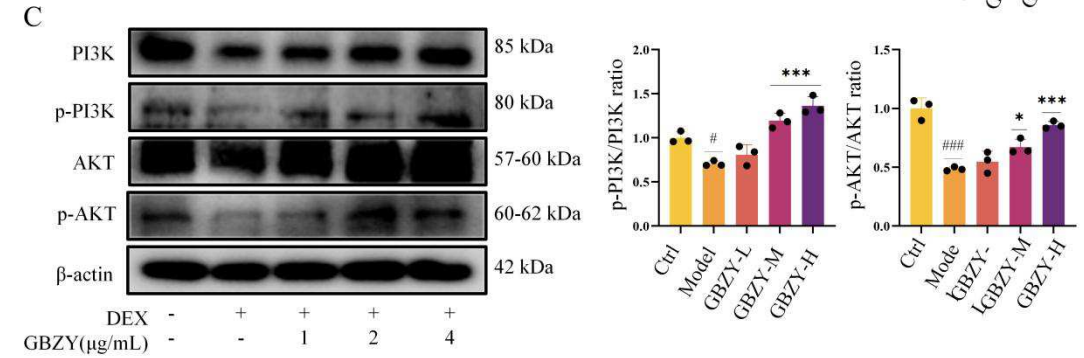
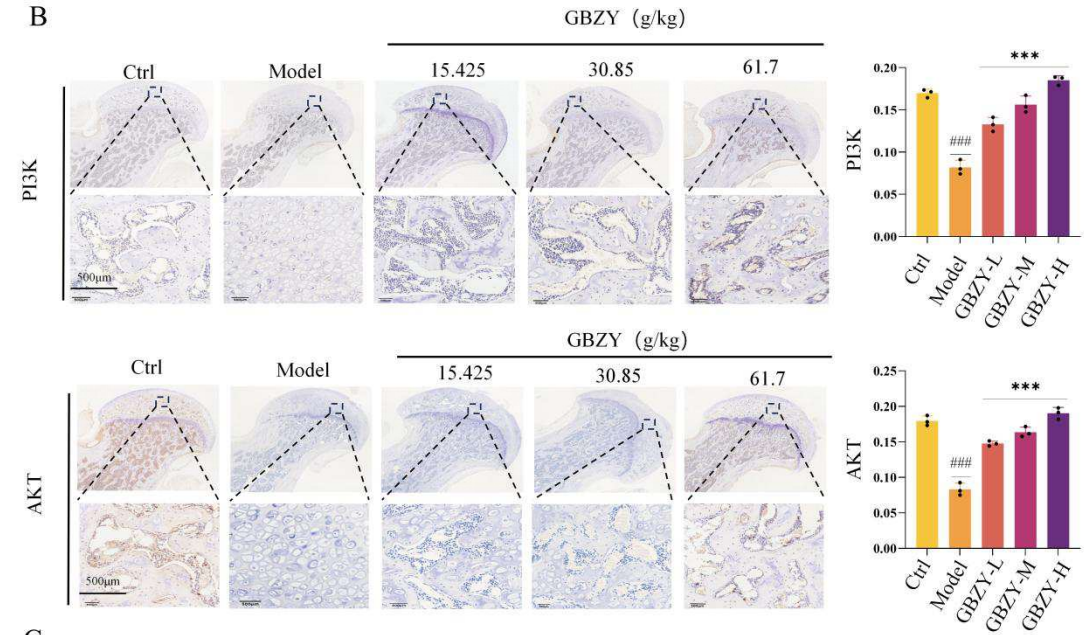
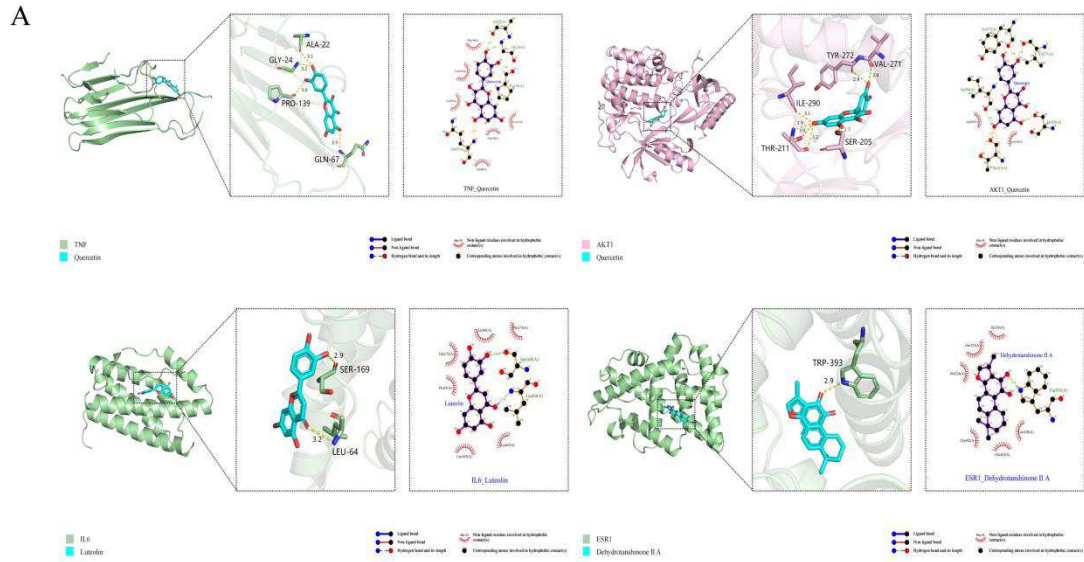


Fig. 5. Molecular docking and pathway validation. A. Molecular docking results. B–C. PI3K/AKT signaling pathway analysis: IHC results (n = 3) including PI3K and AKT (500µm); and WB results (n = 3) including PI3K, AKT, p-PI3K, and p-AKT. Ctrl vs. model, #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001; model vs. GBZY, **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

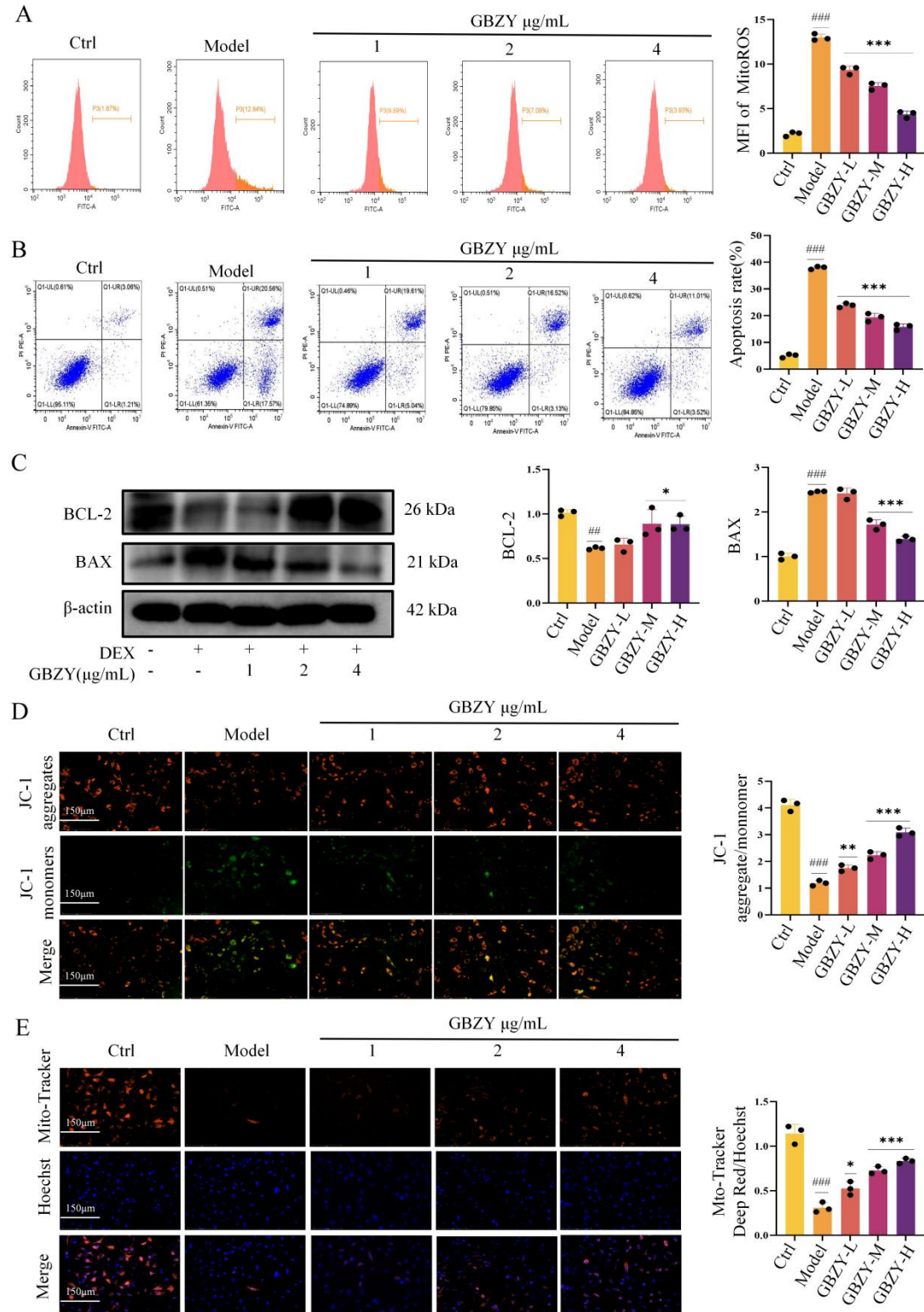


Fig. 6. GBZY ameliorates DEX-induced apoptosis and mitochondrial dysfunction in MC3T3-E1 cells. A. Flow cytometric analysis of ROS levels (n = 3). B. Flow cytometric analysis of cell apoptosis (n = 3). C. WB of apoptosis-related proteins (n = 3), including BCL-2 and BAX. D. JC-1 assay for MMP (n = 3) (150µm). E. MitoTracker Deep Red assay for MMP (n = 3) (150µm). Ctrl vs. model, #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001; model vs. GBZY, **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

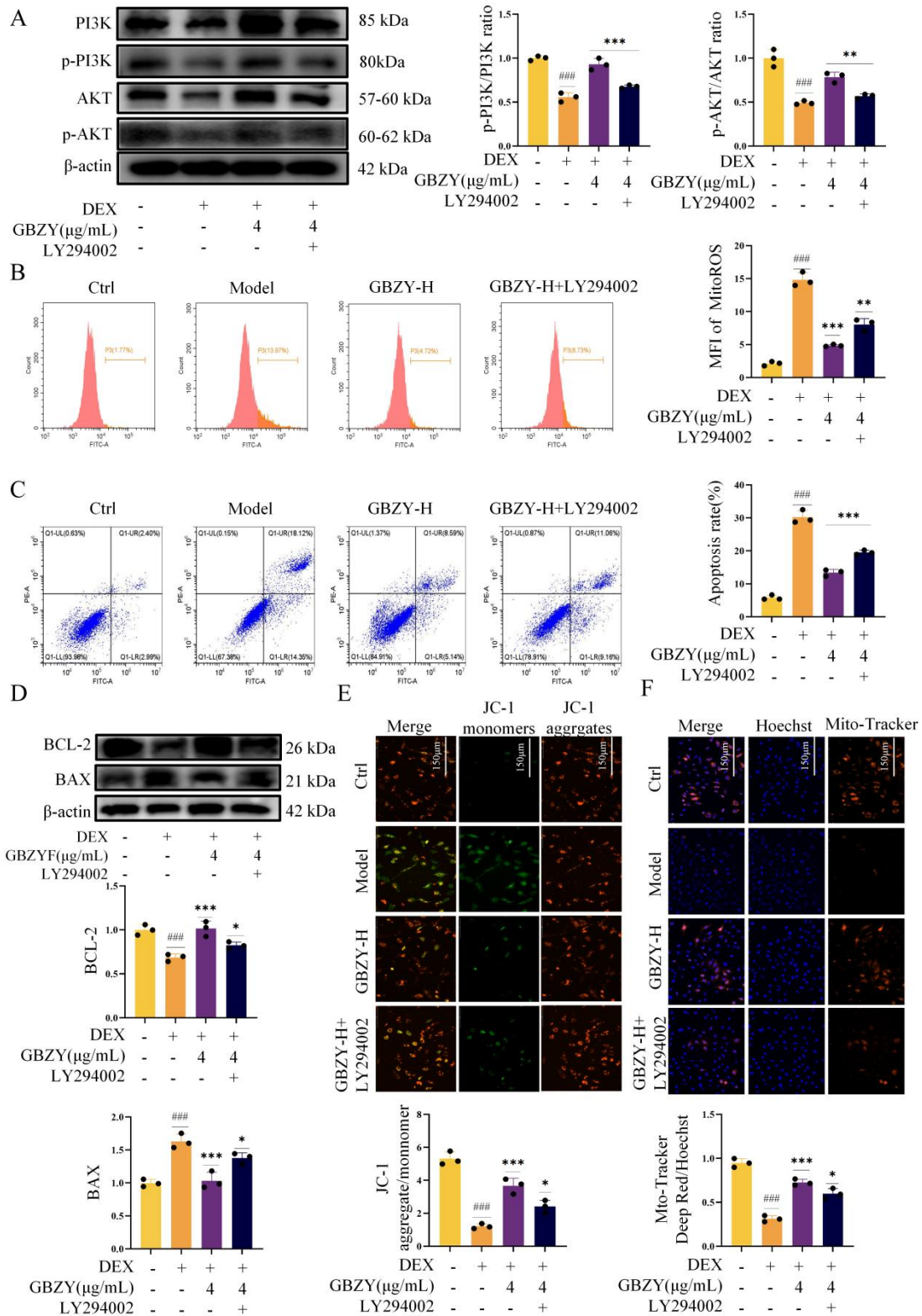


Fig. 7. LY294002 inhibits GBZY-activated PI3K/AKT signaling, impairs mitochondrial function, and increases apoptosis. A. WB of PI3K/AKT signaling pathway proteins (n = 3), including PI3K, AKT, p-PI3K, and p-AKT. B. Flow cytometric analysis of ROS levels (n = 3). C. Flow cytometric analysis of cell apoptosis (n = 3). D. WB of apoptosis-related proteins (n = 3), including BCL-2 and BAX. E. JC-1 assay for MMP (n = 3) (150μm). F. MitoTracker Deep Red assay for MMP (n = 3) (150μm). Ctrl vs. model, #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001; model vs. GBZY-H or GBZY-H vs. GBZY-H+LY, **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

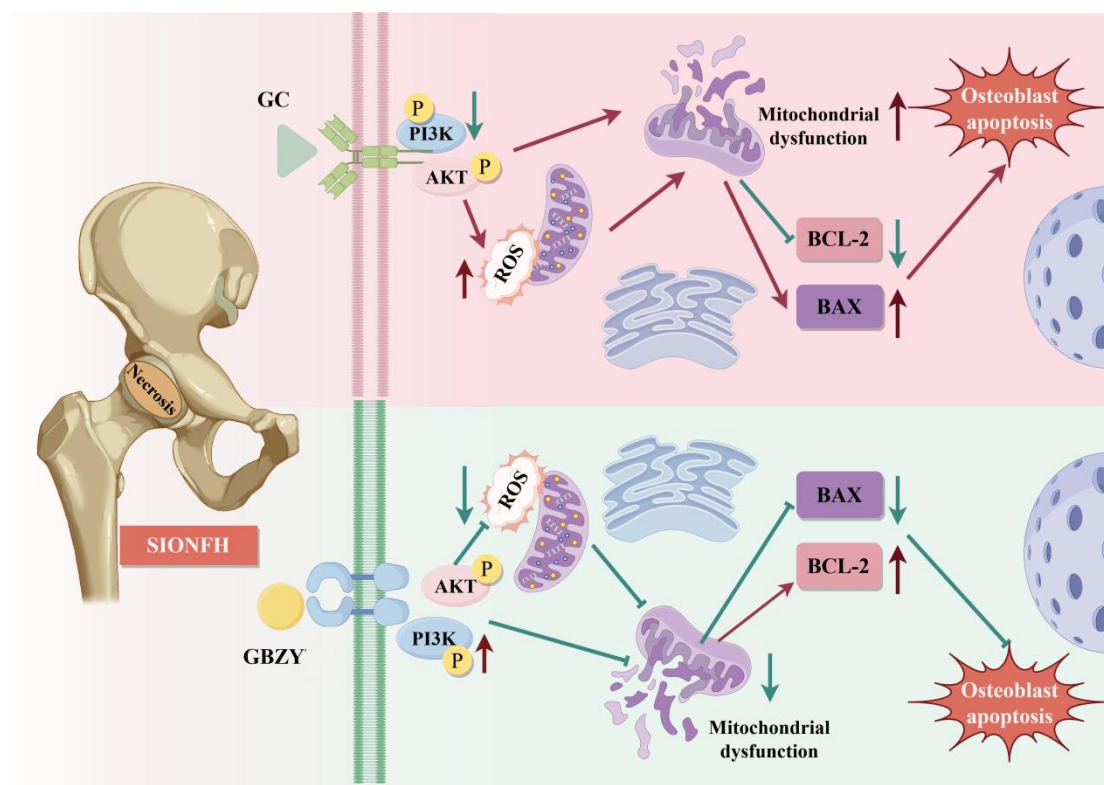


Fig. 8. Schematic illustration of the mechanism of action of GBZY. The core mechanism by which GBZY alleviates SIONFH lies in the activation of the PI3K/AKT pathway, thereby counteracting DEX-induced osteoblast apoptosis through inhibition of the mitochondrial apoptotic pathway.

References

- [1] Liu LH, Zhang QY, Sun W, Li ZR, Gao FQ. Corticosteroid-induced Osteonecrosis of the Femoral Head: Detection, Diagnosis, and Treatment in Earlier Stages. *Chin Med J (Engl)*. 2017;130(21):2601-2607. doi:10.4103/0366-6999.217094.
- [2] Chang C, Greenspan A, Gershwin ME. The pathogenesis, diagnosis and clinical manifestations of steroid-induced osteonecrosis. *J Autoimmun*. 2020;110:102460. doi:10.1016/j.jaut.2020.102460.
- [3] Mont MA, Pivec R, Banerjee S, Issa K, Elmallah RK, Jones LC. High-Dose Corticosteroid Use and Risk of Hip Osteonecrosis: Meta-Analysis and Systematic Literature Review. *J Arthroplasty*. 2015;30(9):1506-1512.e5. doi:10.1016/j.arth.2015.03.036.
- [4] Shigemura T, Nakamura J, Kishida S, et al. Incidence of osteonecrosis associated with corticosteroid therapy among different underlying diseases: prospective MRI study. *Rheumatology (Oxford)*. 2011;50(11):2023-2028. doi:10.1093/rheumatology/ker277.
- [5] Zhang F, Peng W, Wang T, et al. Lnc Tmem235 promotes repair of early steroid-induced

- osteonecrosis of the femoral head by inhibiting hypoxia-induced apoptosis of BMSCs. *Exp Mol Med*. 2022;54(11):1991-2006. doi:10.1038/s12276-022-00875-0.
- [6] Lou Y, Wu J, Zhong Y, Tong P, Du W. Etiology, pathology, and treatment of osteonecrosis of the femoral head in adolescents: A comprehensive review. *Medicine (Baltimore)*. 2024;103(30):e39102. doi:10.1097/MD.00000000000039102.
- [7] Association Related To Circulation Osseous Chinese Microcirculation Society Csm-Arco. *Zhongguo Xue Fu Chong Jian Wai Ke Za Zhi*. 2022;36(11):1319-1326. doi:10.7507/1002-1892.202207134.
- [8] Roth A, Beckmann J, Bohndorf K, et al. S3-Guideline non-traumatic adult femoral head necrosis. *Arch Orthop Trauma Surg*. 2016;136(2):165-174. doi:10.1007/s00402-015-2375-7.
- [9] Yue C, Jin H, Zhang X, et al. Aucubin prevents steroid-induced osteoblast apoptosis by enhancing autophagy via AMPK activation. *J Cell Mol Med*. 2021;25(21):10175-10184. doi:10.1111/jcmm.16954.
- [10] Zheng LW, Wang WC, Mao XZ, Luo YH, Tong ZY, Li D. TNF- α regulates the early development of avascular necrosis of the femoral head by mediating osteoblast autophagy and apoptosis via the p38 MAPK/NF- κ B signaling pathway. *Cell Biol Int*. 2020;44(9):1881-1889. doi:10.1002/cbin.11394.
- [11] Prenek L, Boldizsár F, Kugyelka R, et al. The regulation of the mitochondrial apoptotic pathway by glucocorticoid receptor in collaboration with Bcl-2 family proteins in developing T cells. *Apoptosis*. 2017;22(2):239-253. doi:10.1007/s10495-016-1320-8.
- [12] Sun F, Zhou JL, Liu ZL, Jiang ZW, Peng H. Dexamethasone induces ferroptosis via P53/SLC7A11/GPX4 pathway in glucocorticoid-induced osteonecrosis of the femoral head. *Biochem Biophys Res Commun*. 2022;602:149-155. doi:10.1016/j.bbrc.2022.02.112.
- [13] Wang W, Jiang H, Yu J, Lou C, Lin J. Astaxanthin-mediated Nrf2 activation ameliorates glucocorticoid-induced oxidative stress and mitochondrial dysfunction and impaired bone formation of glucocorticoid-induced osteonecrosis of the femoral head in rats. *J Orthop Surg Res*. 2024;19(1):294. Published 2024 May 14. doi:10.1186/s13018-024-04775-z.
- [14] Chen K, Liu Y, He J, et al. Steroid-induced osteonecrosis of the femoral head reveals enhanced reactive oxygen species and hyperactive osteoclasts. *Int J Biol Sci*. 2020;16(11):1888-1900. Published 2020 Apr 6. doi:10.7150/ijbs.40917.
- [15] Fan Y, Chen Z, Wang H, et al. Isovitexin targets SIRT3 to prevent steroid-induced osteonecrosis of the femoral head by modulating mitophagy-mediated ferroptosis. *Bone Res*. 2025;13(1):18. Published 2025 Jan 26. doi:10.1038/s41413-024-00390-0.
- [16] Xue Y, Cheng X, Ma ZQ, et al. Polystyrene nanoplastics induce apoptosis, autophagy, and steroidogenesis disruption in granulosa cells to reduce oocyte quality and fertility by inhibiting the PI3K/AKT pathway in female mice. *J Nanobiotechnology*. 2024;22(1):460. Published 2024 Aug 1. doi:10.1186/s12951-024-02735-7.
- [17] Li R, Zhang C, Ren J, et al. Remimazolam inhibits apoptosis of endothelial and epithelial cells by activating the PI3K/AKT pathway in acute lung injury. *Int Immunopharmacol*. 2025;147:113949. doi:10.1016/j.intimp.2024.113949.
- [18] Zheng Z, Nan B, Liu C, et al. Inhibition of histone methyltransferase PRMT5 attenuates cisplatin-induced hearing loss through the PI3K/Akt-mediated mitochondrial apoptotic pathway. *J Pharm Anal*. 2023;13(6):590-602. doi:10.1016/j.jpha.2023.04.014.
- [19] Han B, Jiang P, Li Z, et al. Coptisine-induced apoptosis in human colon cancer cells

- (HCT-116) is mediated by PI3K/Akt and mitochondrial-associated apoptotic pathway. *Phytomedicine*. 2018;48:152-160. doi:10.1016/j.phymed.2017.12.027.
- [20] Lee JH, Won YS, Park KH, et al. Celestrol inhibits growth and induces apoptotic cell death in melanoma cells via the activation ROS-dependent mitochondrial pathway and the suppression of PI3K/AKT signaling. *Apoptosis*. 2012;17(12):1275-1286. doi:10.1007/s10495-012-0767-5.
- [21] Zhou L, Pereiro MT, Li Y, et al. Glucocorticoids induce a maladaptive epithelial stress response to aggravate acute kidney injury. *Sci Transl Med*. 2024;16(767):eadk5005. doi:10.1126/scitranslmed.adk5005.
- [22] Shami A, Atzler D, Bosmans LA, et al. Glucocorticoid-induced tumour necrosis factor receptor family-related protein (GITR) drives atherosclerosis in mice and is associated with an unstable plaque phenotype and cerebrovascular events in humans. *Eur Heart J*. 2020;41(31):2938-2948. doi:10.1093/eurheartj/ehaa484.
- [23] Orsi FA, Lijfering WM, Geersing GJ, et al. Glucocorticoid use and risk of first and recurrent venous thromboembolism: self-controlled case-series and cohort study. *Br J Haematol*. 2021;193(6):1194-1202. doi:10.1111/bjh.17388.
- [24] Duan Y, Zhao LJ, Lu YT, Li J, Li SX. Crosstalk between kidney and bones: New perspective for modulating osteoporosis. *Ageing Res Rev*. 2025;109:102776. doi:10.1016/j.arr.2025.102776.
- [25] Lin C, Jiang H, Lou C, et al. Asiatic acid prevents glucocorticoid-induced femoral head osteonecrosis via PI3K/AKT pathway. *Int Immunopharmacol*. 2024;130:111758. doi:10.1016/j.intimp.2024.111758.
- [26] Li L, Zhao S, Leng Z, et al. Pathological mechanisms and related markers of steroid-induced osteonecrosis of the femoral head. *Ann Med*. 2024;56(1):2416070. doi:10.1080/07853890.2024.2416070.
- [27] Meng K, Liu Y, Ruan L, Chen L, Chen Y, Liang Y. Suppression of apoptosis in osteocytes, the potential way of natural medicine in the treatment of osteonecrosis of the femoral head. *Biomed Pharmacother*. 2023;162:114403. doi:10.1016/j.biopha.2023.114403.
- [28] Zhang L, Guan Q, Wang Z, Feng J, Zou J, Gao B. Consequences of Aging on Bone. *Aging Dis*. 2023;15(6):2417-2452. Published 2023 Nov 20. doi:10.14336/AD.2023.1115.
- [29] Zhu X, Qin Z, Zhou M, et al. The Role of Mitochondrial Permeability Transition in Bone Metabolism, Bone Healing, and Bone Diseases. *Biomolecules*. 2024;14(10):1318. Published 2024 Oct 17. doi:10.3390/biom14101318.
- [30] Zheng Y, Zheng Z, Zhang K, Zhu P. Osteonecrosis in systemic lupus erythematosus: Systematic insight from the epidemiology, pathogenesis, diagnosis and management. *Autoimmun Rev*. 2022;21(2):102992. doi:10.1016/j.autrev.2021.102992.
- [31] Gebhard KL, Maibach HI. Relationship between systemic corticosteroids and osteonecrosis. *Am J Clin Dermatol*. 2001;2(6):377-388. doi:10.2165/00128071-200102060-00004.
- [32] Weinstein RS. Glucocorticoid-induced osteonecrosis. *Endocrine*. 2012;41(2):183-190. doi:10.1007/s12020-011-9580-0.
- [33] Zhang X, Feng C, Yuan T, et al. Inhibition of protein disulfide isomerase mitigates steroid-induced osteonecrosis of the femoral head by suppressing osteoclast activity through the reduction of cellular oxidative stress. *Chem Biol Interact*. 2024;404:111263. doi:10.1016/j.cbi.2024.111263.
- [34] Ma T, Wang Y, Ma J, Cui H, Feng X, Ma X. Research progress in the pathogenesis of

- hormone-induced femoral head necrosis based on microvessels: a systematic review. *J Orthop Surg Res.* 2024;19(1):265. Published 2024 Apr 26. doi:10.1186/s13018-024-04748-2.
- [35] Hines JT, Jo WL, Cui Q, et al. Osteonecrosis of the Femoral Head: an Updated Review of ARCO on Pathogenesis, Staging and Treatment. *J Korean Med Sci.* 2021;36(24):e177. Published 2021 Jun 21. doi:10.3346/jkms.2021.36.e177.
- [36] Ding X, Yang J, Wei Y, et al. The Nexus Between Traditional Chinese Medicine and Immunoporosis: Implications in the Treatment and Management of Osteoporosis. *Phytother Res.* 2025;39(4):1826-1846. doi:10.1002/ptr.8397.
- [37] Li W, Yu L, Li W, et al. Prevention and treatment of inflammatory arthritis with traditional Chinese medicine: Underlying mechanisms based on cell and molecular targets. *Ageing Res Rev.* 2023;89:101981. doi:10.1016/j.arr.2023.101981.
- [38] Ouyang W, Meng Y, Guo G, Zhao C, Zhou X. Efficacy and safety of traditional Chinese medicine in the treatment of osteonecrosis of the femoral head. *J Orthop Surg Res.* 2023;18(1):600. Published 2023 Aug 14. doi:10.1186/s13018-023-04086-9.
- [39] Fang S, Wu S, Chen P. Alpha-2-macroglobulin mitigates glucocorticoid-induced osteonecrosis via Keap1/Nrf2 pathway activation. *Free Radic Biol Med.* 2024;225:501-516. doi:10.1016/j.freeradbiomed.2024.09.048.
- [40] Wang P, Wang C, Meng H, et al. The Role of Structural Deterioration and Biomechanical Changes of the Necrotic Lesion in Collapse Mechanism of Osteonecrosis of the Femoral Head. *Orthop Surg.* 2022;14(5):831-839. doi:10.1111/os.13277.
- [41] Zhang J, Cao J, Liu Y, Zhao H. Advances in the Pathogenesis of Steroid-Associated Osteonecrosis of the Femoral Head. *Biomolecules.* 2024;14(6):667. Published 2024 Jun 6. doi:10.3390/biom14060667.
- [42] Huang J, Ma T, Wang C, et al. SOST/Sclerostin impairs the osteogenesis and angiogenesis in glucocorticoid-associated osteonecrosis of femoral head. *Mol Med.* 2024;30(1):167. Published 2024 Sep 28. doi:10.1186/s10020-024-00933-5.
- [43] Wang W, Wu H, Feng S, et al. Tanshinone IIA promotes osteogenic differentiation potential and suppresses adipogenic differentiation potential of bone marrow mesenchymal stem cells. *Mol Med Rep.* 2024;30(4):177. doi:10.3892/mmr.2024.13301.
- [44] Li X, Yang X, Liu Z, et al. Tanshinone IIA Reverses Osteogenic Differentiation of Bone Marrow Mesenchymal Stromal Cells Impaired by Glucocorticoids via the ERK1/2-CREB Signaling Pathway. *Chem Biol Drug Des.* 2025;105(3):e70069. doi:10.1111/cbdd.70069.
- [45] Xie B, Zeng Z, Liao S, Zhou C, Wu L, Xu D. Kaempferol Ameliorates the Inhibitory Activity of Dexamethasone in the Osteogenesis of MC3T3-E1 Cells by JNK and p38-MAPK Pathways. *Front Pharmacol.* 2021;12:739326. Published 2021 Oct 5. doi:10.3389/fphar.2021.739326.
- [46] Feng Y, Dang X, Zheng P, et al. Quercetin in Osteoporosis Treatment: A Comprehensive Review of Its Mechanisms and Therapeutic Potential. *Curr Osteoporos Rep.* 2024;22(3):353-365. doi:10.1007/s11914-024-00868-0.
- [47] Li H, Gong Y, Wang Y, et al. β -Sitosterol modulates osteogenic and adipogenic balance in BMSCs to suppress osteoporosis via regulating mTOR-IMP1-Adipoq axis. *Phytomedicine.* 2025;138:156400. doi:10.1016/j.phymed.2025.156400.
- [48] Kwon SM, Kim S, Song NJ, et al. Antiadipogenic and proosteogenic effects of luteolin, a major dietary flavone, are mediated by the induction of DnaJ (Hsp40) Homolog, Subfamily B,

- Member 1. *J Nutr Biochem*. 2016;30:24-32. doi:10.1016/j.jnutbio.2015.11.013.
- [49] Sun F, Zhou JL, Wei SX, Jiang ZW, Peng H. Glucocorticoids induce osteonecrosis of the femoral head in rats via PI3K/AKT/FOXO1 signaling pathway. *PeerJ*. 2022;10:e13319. Published 2022 May 3. doi:10.7717/peerj.13319.
- [50] Xue XH, Feng ZH, Li ZX, Pan XY. Salidroside inhibits steroid-induced avascular necrosis of the femoral head via the PI3K/Akt signaling pathway: In vitro and in vivo studies. *Mol Med Rep*. 2018;17(3):3751-3757. doi:10.3892/mmr.2017.8349.
- [51] Mustafa M, Ahmad R, Tantry IQ, et al. Apoptosis: A Comprehensive Overview of Signaling Pathways, Morphological Changes, and Physiological Significance and Therapeutic Implications. *Cells*. 2024;13(22):1838. Published 2024 Nov 6. doi:10.3390/cells13221838.
- [52] Z. Li, S. Li, W. Chen, et al. Mitochondrial dysfunction: Related diseases, influencing factors, and detection. *Interdiscip. Med*. 2025, 3, e20250005. <https://doi.org/10.1002/INMD.20250005>.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementaryfile.docx](#)