Baicalin attenuates dexamethasone-induced apoptosis of bone marrow mesenchymal stem cells by activating the hedgehog signaling pathway

Bin Jia[1,2], Yaping Jiang[3], Yao Yao[2], Yingxing Xu[1,2], Yingzhen Wang[1], Tao Li[1]

[1]Department of Joint Surgery, The Affiliated Hospital of Qingdao University, Qingdao, Shandong 266003, China;
[2]Medical Department, Qingdao University, Qingdao, Shandong 266071, China;
[3]Department of Oral Implantology, The Affiliated Hospital of Qingdao University, Qingdao, Shandong 266003, China.

Abstract

Background: Perturbations in bone marrow mesenchymal stem cell (BMSC) differentiation play an important role in steroid-induced osteonecrosis of the femoral head (SONFH). At present, studies on SONFH concentrate upon the balance within BMSC osteogenic and adipogenic differentiation. However, BMSC apoptosis as well as proliferation are important prerequisites in their differentiation. The hedgehog (HH) signaling pathway regulates bone cell apoptosis. Baicalin (BA), a well-known compound in traditional Chinese medicine, can affect the proliferation and apoptosis of numerous cell types via HH signaling. However, the potential role and mechanisms of BA on BMSCs are unclear. Thus, we aimed to explore the role of BA in dexamethasone (Dex)-induced BMSC apoptosis in this study.

Methods: Primary BMSCs were treated with 10^{-6} mol/L Dex alone or with 5.0 μmol/L, 10.0 μmol/L, or 50.0 μmol/L BA for 24 hours followed by co-treatment with 5.0 μmol/L, 10.0 μmol/L, or 50.0 μmol/L BA and 10^{-6} mol/L Dex. Cell viability was assayed through the Cell Counting Kit-8 (CCK-8). Cell apoptosis was evaluated using Annexin V-fluorescein isothiocyanate/propidium iodide (PI) staining followed by flow cytometry. The imaging and counting, respectively, of Hochest 33342/PI-stained cells were used to assess the morphological characteristics and proportion of apoptotic cells. To quantify the apoptosis-related proteins (e.g., apoptosis regulator BAX [Bax], B-cell lymphoma 2 [Bcl-2], caspase-3, and cleaved caspase-3) and HH signaling pathway proteins, western blotting was used. A HH-signaling pathway inhibitor was used to demonstrate that BA exerts its anti-apoptotic effects via the HH signaling pathway.

Results: The results of CCK-8, Hoechst 33342/PI-staining, and flow cytometry showed that BA did not significantly promote cell proliferation (CCK-8: 0 μmol/L, 100%; 2.5 μmol/L, 98.58%; 5.0 μmol/L, 95.18%; 10.0 μmol/L, 98.11%; 50.0 μmol/L, 99.38%, $F = 2.33$, $P > 0.05$), but it did attenuate the effect of Dex on apoptosis (Hoechst 33342/PI-staining: Dex+ 50.0 μmol/L BA, 12.27% *vs*. Dex, 39.27%, $t = 20.62$; flow cytometry: Dex + 50.0 μmol/L BA, 12.68% *vs*. Dex, 37.43%, $t = 11.56$; Both $P < 0.05$). The results of western blotting analysis showed that BA reversed Dex-induced apoptosis by activating the HH signaling pathway, which down-regulated the expression of Bax, cleaved-caspase 3, and suppressor of fused (SUFU) while up-regulating Bcl-2, sonic hedgehog (SHH), and zinc finger protein GLI-1 (GLI-1) expression (Bax/Bcl-2: Dex+ 50.0 μmol/L BA, 1.09 *vs*. Dex, 2.76, $t = 35.12$; cleaved caspase-3/caspase-3: Dex + 50.0 μmol/L BA, 0.38 *vs*. Dex, 0.73, $t = 10.62$; SHH: Dex + 50.0 μmol/L BA, 0.50 *vs*. Dex, 0.12, $t = 34.01$; SUFU: Dex+ 50.0 μmol/L BA, 0.75 *vs*. Dex, 1.19, $t = 10.78$; GLI-1: Dex+ 50.0 μmol/L BA, 0.40 *vs*. Dex, 0.11, $t = 30.68$. All $P < 0.05$).

Conclusions: BA antagonizes Dex-induced apoptosis of human BMSCs by activating the HH signaling pathway. It is a potential candidate for preventing SONFH.

Keywords: Baicalin; Dexamethasone; Apoptosis; Bone marrow mesenchymal stem cell; Hedgehog

Introduction

Bone marrow mesenchymal stem cells (BMSCs) are multipotent, with the ability to differentiate into adipocytes, chondrocytes, or osteoblasts.[1-3] Many diseases, such as osteoporosis or steroid-induced osteonecrosis of the femoral head (SONFH), occur because the balance between osteogenic and adipogenic differentiation is disrupted.[4,5] BMSC apoptosis and proliferation are important prerequisites in their differentiation. Yet, past studies on SONFH have focused on the unbalanced differentiation of BMSCs while ignoring their apoptosis and proliferation.

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Correspondence to: Dr. Tao Li, Department of Joint Surgery, The Affiliated Hospital of Qingdao University, 59 Haier Road, Laoshan District, Qingdao, Shandong 266003, China
E-Mail: qdult@qdu.edu.cn

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Dexamethasone (Dex) is one of the common synthetic glucocorticoids, with the effects of immunosuppression and anti-inflammation.[6] The long-term use of hormonal drugs may lead to femoral head necrosis. Indeed, low concentrations of Dex can induce BMSCs to differentiate into bone and fat,[7,8] while high concentrations (10^{-6} mol/L) can promote and inhibit, respectively, BMSC apoptosis and proliferation.[9,10] This effect likely contributes to hormonal-drug-induced SONFH. In a prior study,[11] we revealed that certain non-coding RNAs may be related to Dex-induced apoptosis of BMSCs. Here, to explore drug and gene-level treatments, we attempt to elucidate the molecular signaling pathways related with Dex-induced BMSC apoptosis.

Baicalin (BA) is the main active ingredient extracted from *Scutellaria baicalensis*. Because of its anti-tumor, anti-oxidation, and anti-inflammatory effects,[12-14] it has been broadly applied in traditional Chinese medicine. In orthopedics, BA can be used to treat osteoarthritis due to its anti-inflammatory effects on chondrocytes.[15-17] Further, it can reduce apoptosis by enhancing chondrocyte autophagy protection[18] and ameliorating endoplasmic reticulum stress.[19] BA has been reported to increase mesenchymal stem cell (MSC) survival after transplantation by reducing apoptosis,[20] as well as the survival of cardiomyocytes treated with H_2O_2.[21] The hedgehog (HH) signaling pathway is highly conserved; it regulates embryonic development and adult tissue homeostasis.[22] Its abnormal activation can promote tumorigenesis and tumor development.[23-26] BA can reduce apoptosis via the HH signaling pathway.[27] As reported in two studies,[28,29] activating the HH signaling pathway reduces the apoptosis of MSCs. Therefore, this study aimed to explore whether BA can block Dex-induced BMSC apoptosis through activating the HH signaling pathway.

Based on prior research, we hypothesize that BA reduces Dex-induced BMSC apoptosis through activating the HH signaling pathway. Therefore, we conducted this experiment to verify this hypothesis.

Methods

Ethical approval

Human BMSC (hBMSC) primary cultures were obtained from three patients (aged 45, 47, and 52 years) with fractures of femoral neck and received total hip arthroplasty at the Affiliated Hospital of Qingdao University.

All patients signed informed consent forms. This study was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University (No. QYFYWZLL26792).

Human BMSC isolation, cultivation, and treatment

hBMSCs were extracted from bone marrow tissue as described in a previous study.[30] They were cultivated in culture flasks in low glucose Dulbecco's Modified Eagle Medium containing 1% fetal bovine serum. The culture flasks were placed in a humidified incubator (ThermoFish Scientific, Massachusetts, USA). When confluence reached 8% to 9%, the cells were digested with trypsin and passaged into new culture flasks at a ratio of 1:2.

Primary hBMSCs were either treated with 10^{-6} mol/L Dex alone or with BA (5.0 μmol/L, 10.0 μmol/L, and 50.0 μmol/L, respectively) for 24 hours followed by co-treatment with 5.0 μmol/L, 10.0 μmol/L, or 50.0 μmol/L BA and 10^{-6}mol/L Dex. Depending on the experimental condition, the cells were treated with drugs for a different number of days.

Cell phenotype identification

Each time the primary hBMSCs were extracted, they were subjected to phenotype identification. First, third-generation hBMSCs were re-suspended in 100 μL phosphate buffered saline (PBS) at a concentration of 1×10^6 cells/mL. The cell suspensions were then incubated with cluster of differentiation ^{34}(CD34)-phycoerythrin (PE), CD45-PE, CD73-fluorescein isothiocyanate (FITC), and CD90-FITC for 45 minutes with the condition of room temperature and in darkness. All antibodies were purchased from BD Biosciences (Franklin Lake, New Jersey, United States). After washing twice with PBS, those single-stained cells were re-suspended in 100 μL PBS. The negative control was unstained cells. The five groups of cell samples were processed using flow cytometry (Apogee A50-MICRO flow cytometer, Apogee, London, UK).

Osteogenic and adipogenic differentiation of hBMSCs

Third-generation hBMSCs were inoculated into six-well plates. To induce osteogenic differentiation, when cell confluence reached 60%, the complete medium would be replaced with an osteogenic medium (Fuyuanbio, Shanghai, China) and cultured for 14 days. To induce adipogenic differentiation, when cell confluence reached 90%, the complete medium would be replaced with an adipogenic induction medium (Fuyuanbio) and cultured for 21 days. The cells were washed with PBS twice, followed by fixation with formalin (10%). Afterward, the cells were stained with alkaline phosphatase (Hat Biotechnology Co. Ltd., Xi'an, China) or Oil Red O (Solarbio, Beijing, China) for 30 minutes.

Cell viability analysis

For 96-well plates, 5×10^3 cells per well were inoculated, and further processed after 24 hours according to their experimental groups. Cell viability was determined by Cell Counting Kit-8 (CCK-8) assay kit (Solarbio). Complete medium (100 μL) as well as CCK-8 reagent (10 μL) were both added to each well. With the incubation of 1 to 4 hours in a cell incubator, the absorbance was measured at 450 nm by a microplate reader (Tecan, Austria). Notably, the experiment was repeated three times.

Assessment of apoptotic morphology in Dex-treated hBMSCs

For 24-well plates, a total of 5×10^4 cells per well were inoculated for 24 hours, followed by further procession based on their experimental group. Hoechst 33342/propidium iodide (PI) Kit (Solarbio) was adopted for

the assessment of apoptotic cells' morphology following the manufacturer's instructions. Apoptotic cells that displayed morphological characteristics under a fluorescence microscope, such as chromosome aggregation, nuclear division, and nuclear fragmentation, were identified and counted. Red and blue hyper-fluorescence could be observed in necrotic cells. The experiment was repeated three times.

Flow cytometry analysis of apoptotic cells

Third-generation cells were cultured in six-well plates until the confluence reached approximately 80%. The cells were subsequently treated with different drugs according to their assigned group. After staining with an AnnexinV-FITC/PI apoptosis detection kit (Absin Biotechnology Co. Ltd., Shanghai, China), an Apogee A50-MICRO flow cytometer (Apogee) was used to quantify the percentage of apoptotic cells. The experiment was repeated three times.

Western blotting analysis

To extract total protein, pre-chilled radioimmunoprecipitation assay (RIPA) lysis buffer (Solarbio) having 1% protease inhibitor (MedchemEx-press) was applied to lyse the treated hBMSCs. The bicinchoninic acid protein detection kit (Meilunbio, Dalian, China) was used to determine protein concentration. Protein loading buffer (EpiZyme, Shanghai, China) was added to the extracted total protein solution at a ratio of 1:4, which was then heated to 100°C for 10 minutes. Depending on the molecular weight of the target protein, 6.0% or 12.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (EpiZyme) was used to separate the protein samples (30 μg). Next, the proteins were electro-transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore-Sigma, Shanghai, China). Subsequently, those membranes were placed in tris-buffered saline with Tween 20 (TBST) (Solarbio) containing 5% skimmed milk powder for 1.5 hours. After washed thrice with TBST, PVDF membranes were first incubated with the primary antibody at the temperature of 4°C overnight and then the corresponding secondary antibodies for 1.5 hours. The PVDF membranes were washed three times with TBST. The target bands were visualized with ECL-PLUS reagent (MilliporeSigma), and further scanned by the BioSpectrum imaging system (ThermoFisher Scientific, Massachusetts, USA). ImageJ software (version 1.52; NIH, Bethesda, USA) was used for quantifying gray values of the target bands; the results were normalized to glyceraldehyde 3-phosphate dehydro-genase (GAPDH). The experiment was repeated three times.

The primary antibodies included: rabbit anti-human sonic hedgehog (SHH, 2207T), suppressor of fused (SUFU; 2522S), zinc finger protein GLI-1 (GLI-1; 3538T), apoptosis regulator BAX (Bax; 5023T), B-cell lymphoma 2 (Bcl-2; 4223T), caspase-3 (14220T), and cleaved caspase-3 (14220T). They were all purchased from Cell Signaling Technologies (Danvers, USA). The GAPDH primary antibody as well as all secondary antibodies were provided by Elabscience (Shanghai, China). The antibody dilution solution (Boster Biological Technology, Shanghai, China) was used to dilute all antibodies according to the manufacturer's instructions.

Statistical analysis

All data were analyzed using SPSS 26.0 software (IBM, New York, USA). One-way analysis of variance (ANOVA) was carried out for the comparison among groups, while the independent Student's t test was used to compare between two groups. The data were described as the mean ± standard deviation. P values < 0.05 were considered to be statistically significant. Figures were created by GraphPad Prism 8 software (GraphPad, Inc. CA, USA).

Results

hBMSC identification

After three generations of culturing, the bone-marrow extracted cells displayed a spindle shape [Figure 1A]. Following the induction of osteogenesis or adipogenesis, respectively, alkaline phosphatase or Oil Red O staining was used to determine the cells' ability to differentiate into osteoblasts or adipocytes [Figures 1B and C]. The flow cytometry detection of cell surface markers revealed that most cells expressed typical BMSC markers: CD73 (95.50%) and CD90 (92.00%); few cells expressed atypical markers: CD34 (0.22%) and CD45 (0.24%) [Figure 1D].

Role of BA in cell viability

BA's molecular formula is given in Figure 2A. A complete medium containing BA at 2.5 μmol/L, 5.0 μmol/L, 10.0 μmol/L, 50.0 μmol/L, or 100.0 μmol/L was used to cultivate hBMSCs for 6 days. BA at concentrations of 2.5 μmol/L, 5.0 μmol/L, 10.0 μmol/L, 50.0 μmol/L had almost no effect on cell viability ($P > 0.05$), while BA at 100.0 μmol/L significantly inhibited hBMSC viability ($P < 0.05$). Concurrently, BA was unable to promote hBMSC proliferation [Figure 2B]. Based on these results, BA at concentrations of 5.0 μmol/L, 10.0 μmol/L, or 50.0 μmol/L were used in the following experiments.

BA reverses Dex-induced inhibition of hBMSC proliferation

CCK-8 analysis was used to evaluate hBMSC proliferation under different treatment conditions. When compared to the control group, hBMSCs in the other groups exhibited a decrease in proliferation ($P < 0.05$). However, when compared to the Dex group, cells treated with BA demonstrated a concentration-dependent increase in proliferation, whereby proliferation increased with increasing BA concentrations ($P < 0.05$; Figures 2C and D). These results indicate that BA can attenuate the inhibitory effects of high doses of Dex (10^{-6} mol/L) on hBMSC proliferation.

BA blocks Dex-induced hBMSC apoptosis

High concentrations of Dex (10^{-6} mol/L) can cause hBMSC apoptosis. Apoptotic cells display chromosome aggregation, nuclear division, and nuclear fragmentation.

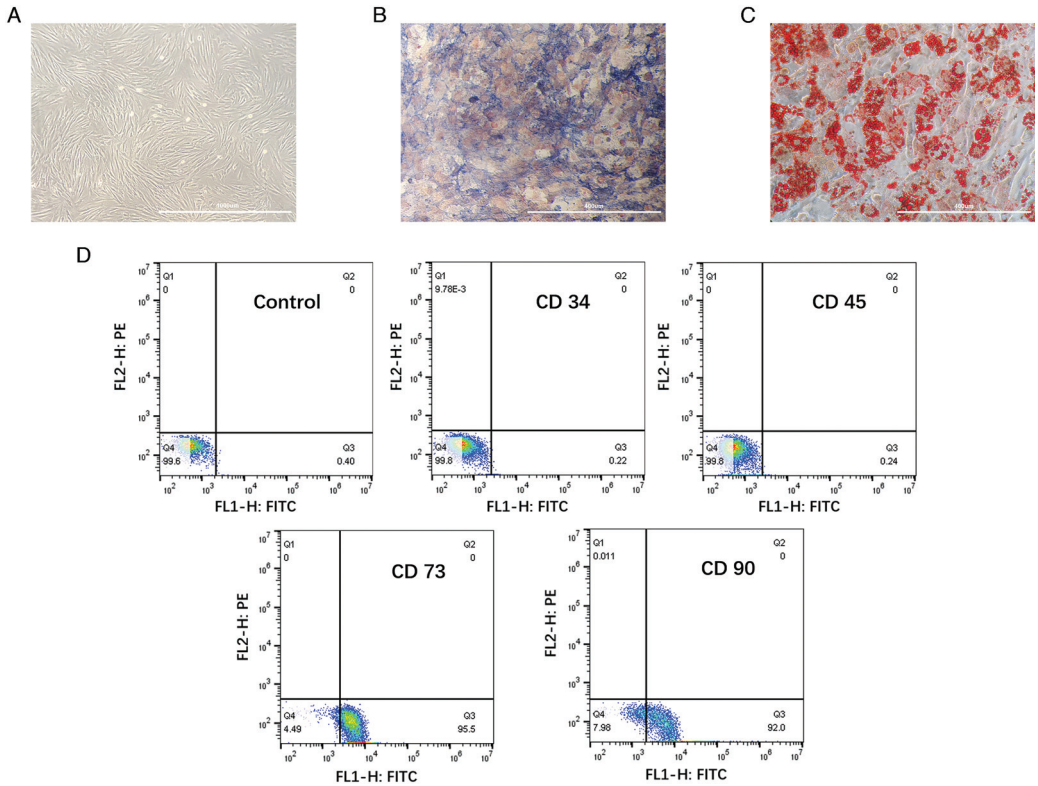

Figure 1: hBMSC verification by morphology and flow cytometry. (A) hBMSC morphology (scale bar: 1000 μm). (B) ALP staining (scale bar: 400 μm). (C) Oil Red O staining (scale bar: 400 μm). (D) Identification of hBMSC surface markers by flow cytometry. ALP: Alkaline phosphatase; FITC: Fluorescein isothiocyanate; hBMSC: Human bone marrow mesenchymal stem cell; Q1: Necrotic cells, Q2: Late apoptotic cells, Q3: Early apoptotic cells, Q4: Normal cells.

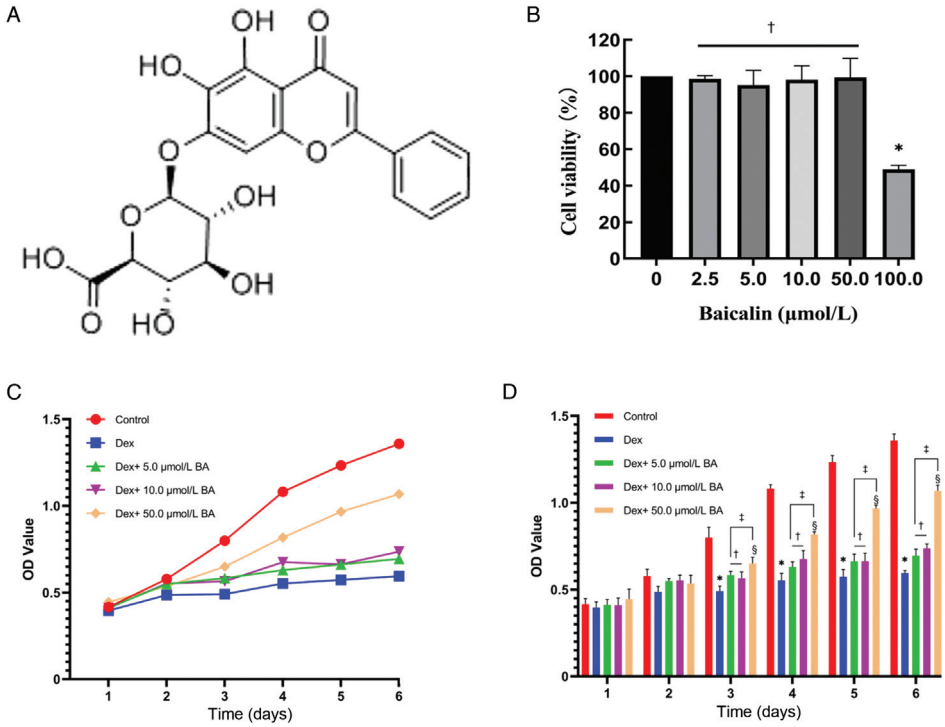

Figure 2: Effect of BA on hBMSC viability. (A) BA chemical formula. (B) hBMSCs were cultured in the presence of differing BA concentrations, and cell viability was measured 6 days later. BA <50.0 μmol/L did not affect cell viability, while BA at 100.0 μmol/L had a significant inhibitory effect. $P < 0.05$ compared to the 0 μmol/L BA group, $^{\dagger}P > 0.05$ compared to the 0 μmol/L BA group. (C) The viability of cells in each group following 6 days of treatment. BA alleviated the inhibitory effect of Dex on hBMSC proliferation. (D) Higher concentrations had stronger effects than lower concentrations. (D) $^{*}P < 0.05$ compared to the control group, $^{\dagger}P > 0.05$ compared to the Dex + 5.0 μmol/L BA group, $^{\ddagger}P < 0.05$ compared to the Dex group, $^{\S}P < 0.05$ compared to the Dex + 5.0 μmol/L BA group. BA: Baicalin; Dex: Dexamethasone; hBMSC: Human bone marrow mesenchymal stem cell; OD: Optical density.

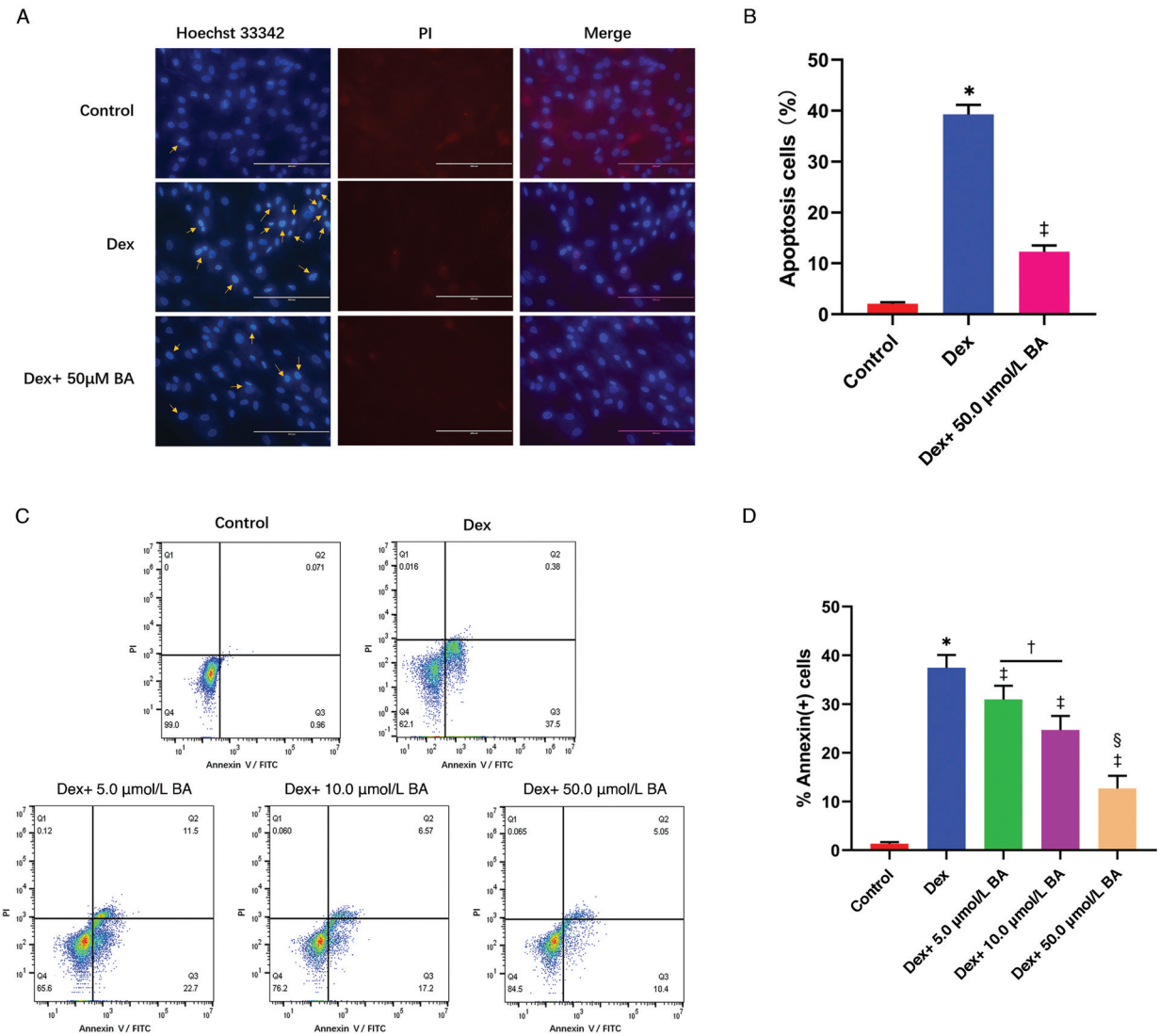

Figure 3: BA attenuated Dex-induced hBMSC apoptosis. (A) Hoechst 33342/PI staining of hBMSCs. Morphology of the stained cells (scale bar: 200 μm). The yellow arrow indicates cells with apoptotic characteristics. (B)The proportion of apoptotic cells following cell counting. (C) Apoptosis was detected using flow cytometry. Flow cytometric analysis of cells with Annexin V-FITC and PI staining. Q1: Necrotic cells, Q2: Late apoptotic cells, Q3: Early apoptotic cells, Q4: Normal cells. (D) The proportion of apoptotic cells in each group. $^{*}P < 0.05$ compared to the control group, $^{\dagger}P > 0.05$ compared to the Dex + 5.0 μmol/L BA group, $^{\ddagger}P < 0.05$ compared to the Dex group, $^{\S}P < 0.05$ compared to the Dex + 5.0 μmol/L BA group. BA: Baicalin; Dex: Dexamethasone; FITC: Fluorescein isothiocyanate; hBMSC: Human bone marrow mesenchymal stem cell; PI: Propidium iodide.

After staining with Hoechst 33342/PI, cells were observed under a fluorescence microscope, where it was found that BA reduced the proportion of apoptotic cells caused by Dex ($P < 0.05$; Figures 3A and B).

Flow cytometry was carried out for the evaluation of hBMSC apoptosis under the different treatments. When compared to the control group, hBMSCs in the other groups exhibited an increase in apoptosis ($P < 0.05$). However, in contrast with the Dex group, hBMSC apoptosis in BA-treated cells was decreased in a concentration-dependent manner; that is, it decreased with increasing BA concentrations [Figures 3C and D].

The degree of apoptosis was further examined at the molecular level. Western blotting was used to quantify the expression of apoptosis-related proteins such as Bax, Bcl-2, caspase-3, and cleaved caspase-3 in hBMSCs. High doses of Dex (10^{-6} mol/L) increased the expression level of apoptosis related proteins, Bax, Bcl-2, cleaved caspase-3, compared to Dex group ($P < 0.05$; Figures 4A and B). The changes in the expression of these apoptosis-related molecules depended on BA concentration [Figure 4B]. Altogether, these data indicate that BA can inhibit Dex-induced apoptosis.

BA reverses Dex-induced apoptosis through the activation of the HH signaling pathway

In addition to detecting apoptosis-related molecules, we used western blotting to assay several proteins of the HH signaling pathway. In hBMSCs treated with Dex (10^{-6}mol/L), SHH and GLI-1 protein levels were significantly reduced while SUFU expression was increased (all $P < 0.05$). These results imply that Dex inhibits the HH signaling pathway. When we treated the cells with BA,

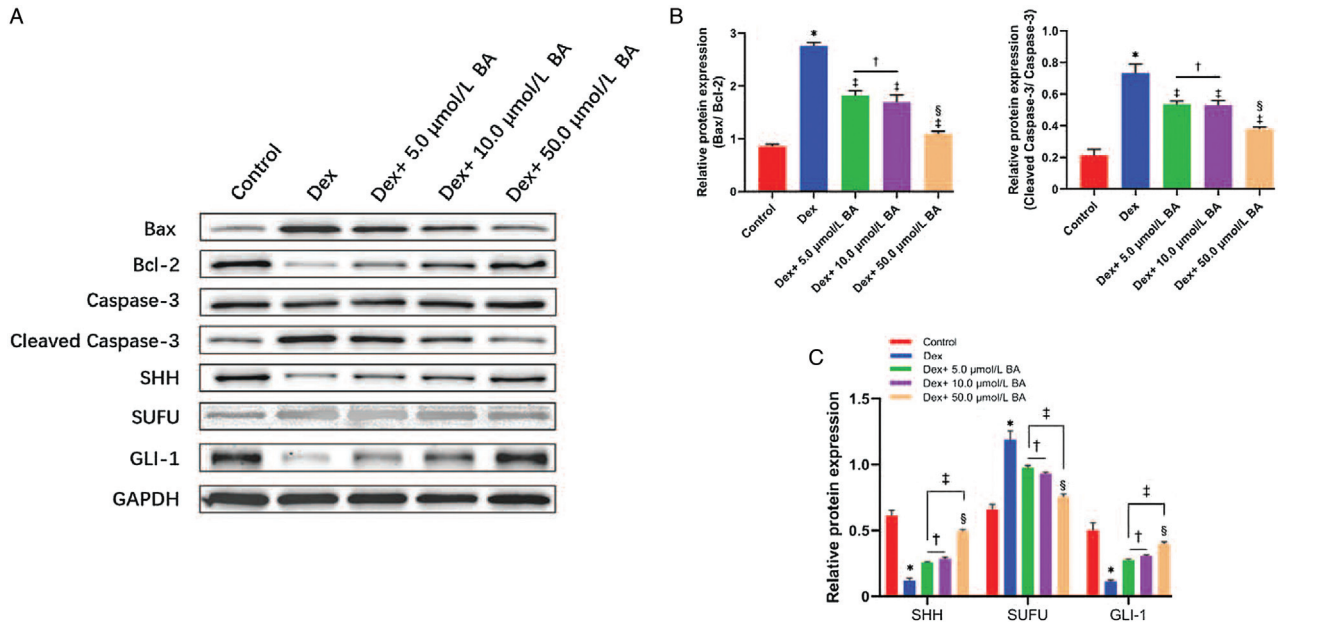

Figure 4: In ameliorating Dex-induced hBMSC apoptosis, BA activates the HH signaling pathway. Western blotting analysis revealed (A) the protein expression levels of Bax, Bcl-2, caspase-3, cleaved caspase-3, SHH, SUFU, and GLI-1 in each group. (B) The relative expression of apoptosis-related proteins in each group. (C) The relative expression levels of HH-signaling-pathway-related proteins in each group. $^{*}P < 0.05$ compared to the control group, $^{\dagger}P > 0.05$ compared to the Dex + 5.0 μmol/L BA group, $^{\ddagger}P < 0.05$ compared to the Dex group, $^{\S}P < 0.05$ compared to the Dex + 5.0 μmol/L BA group. BA: Baicalin; Bax: Apoptosis regulator BAX; Bcl-2: B-cell lymphoma 2; Dex: Dexamethasone; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; GLI-1: Zinc finger protein GLI-1; hBMSC: Human bone marrow mesenchymal stem cell; HH: Hedgehog; SHH: Sonic hedgehog, SUFU: Suppressor of fused.

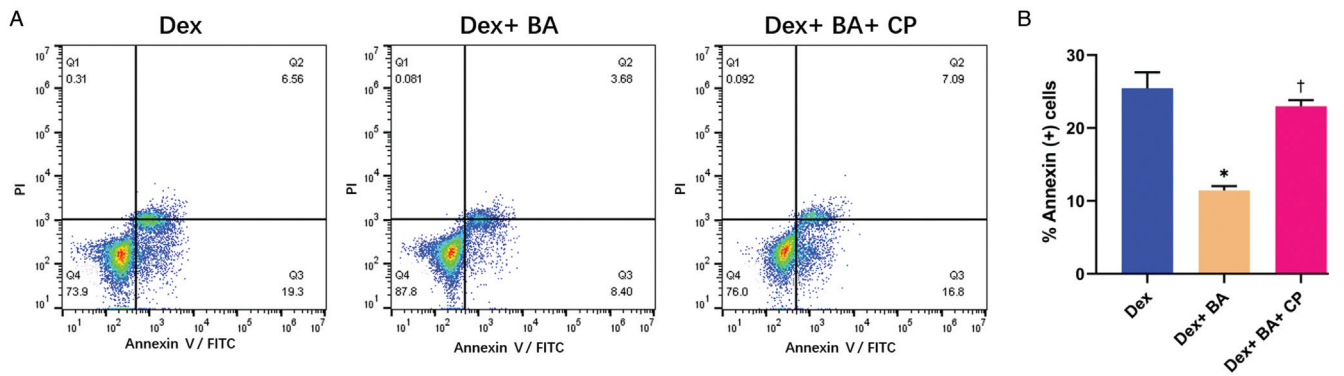

Figure 5: A HH signaling pathway inhibitor was used to demonstrate that BA inhibits Dex-induced apoptosis of hBMSCs via the HH signaling pathway. (A) Flow cytometric analysis of cells stained with Annexin V-FITC and PI. (B) The proportion of apoptotic cells in each group. $^{*}P < 0.05$ compared to the Dex group, $^{\dagger}P < 0.05$ compared to the Dex + BA group. BA: Baicalin; CP: Cyclopamine; Dex: Dexamethasone; FITC: Fluorescein isothiocyanate; hBMSCs: Human bone marrow mesenchymal stem cells; HH: Hedgehog; PI: Propidium iodide. Q1: Necrotic cells, Q2: Late apoptotic cells, Q3: Early apoptotic cells, Q4: Normal cells.

SHH and GLI-1 protein expression increased, while SUFU expression decreased (all $P < 0.05$; Figures 4A and C).

Notably, when cyclopamine (CP), a HH signaling pathway inhibitor (MCE, Shanghai, China), and BA were used concurrently to treat Dex-induced apoptosis of hBMSCs, the flow cytometry analysis revealed that the apoptosis rate of the Dex + BA + CP group was significantly higher than the Dex + BA group ($P < 0.05$; Figures 5A and B). Moreover, the expression of HH-signaling-pathway-related proteins indicated that CP can inhibit the HH signaling pathway [Figures 6A and C] and significantly weaken the anti-apoptotic effects of BA. We found that in hBMSCs stimulated by Dex, the effects of BA on the expression levels of Bax, Bcl-2, and cleaved caspase-3 were all antagonized by CP (all $P < 0.05$; Figures 6A and B). Altogether, these findings indicate that BA exerts its inhibitory effects on Dex-induced apoptosis via the HH signaling pathway.

Discussion

The value of BMSCs lies in their self-renewing and multidirectional differentiation abilities. The understanding of the mechanisms underlying their abnormal function may help improve the treatment of related orthopedic diseases.[4,31] Glucocorticoids are widely used in clinical treatment, but their long-term use can cause SONFH. SONFH is a common orthopedic disease; however, its only treatment is surgery as no drugs are available. Based on previous studies, we inferred that high concentration of

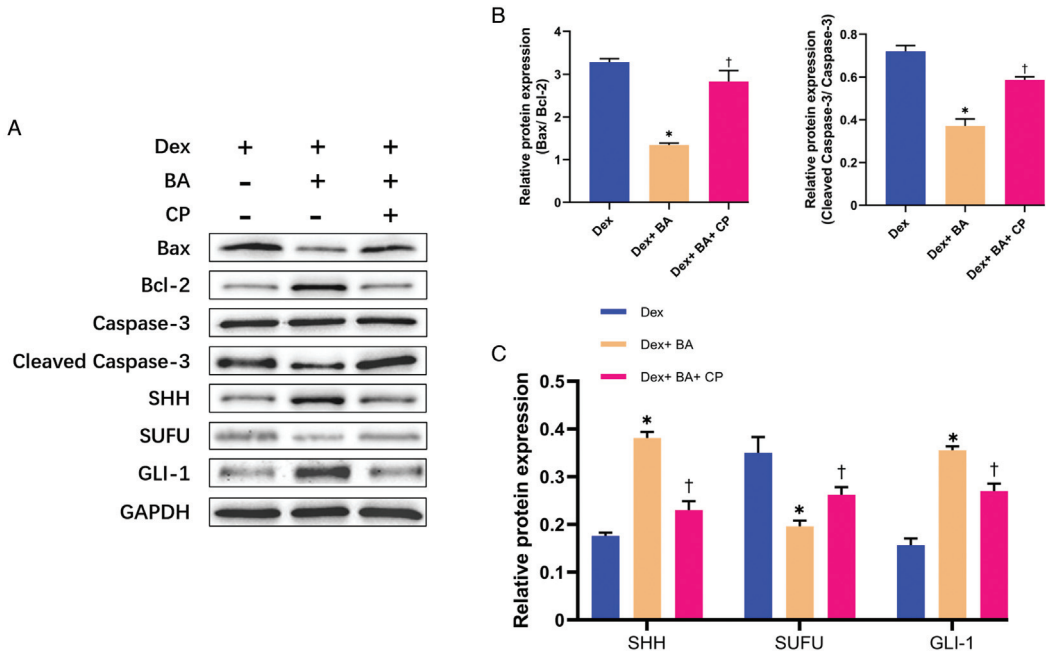

Figure 6: At the molecular level, BA reduces the Dex-induced apoptosis of hBMSCs via the HH signaling pathway. The results of the western blotting analysis revealed (A) the protein expression levels of Bax, Bcl-2, caspase-3, cleaved caspase-3, SHH, SUFU, and GLI-1 in each group. (B) The relative expression of apoptosis-related proteins in each group. (C) The relative expression of HH-signaling-pathway-related proteins in each group. $^{*}P < 0.05$ compared to the Dex group, $^{\dagger}P < 0.05$ compared to the Dex + BA group. BA: Baicalin, Bax: Apoptosis regulator BAX; Bcl-2: B-cell lymphoma 2; CP: Cyclopamine; Dex: Dexamethasone; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; GLI-1: Zinc finger protein GLI-1; hBMSCs: Human bone marrow mesenchymal stem cells; HH: Hedgehog; SHH: Sonic hedgehog; SUFU: Suppressor of fused.

glucocorticoids may cause BMSC apoptosis, which may be a reason for the occurrence of SONFH. Dex, a common glucocorticoid, at high concentrations (10^{-6} mol/L) can promote the apoptosis and inhibit the proliferation of BMSCs.[9,10,32] Therefore, in this study, 10^{-6} mol/LDex was used to induce hBMSC apoptosis to simulate the microenvironment of cells in SONFH. Exploring a compound that can counteract the negative effects of glucocorticoids may be an effective way to prevent or treat SONFH.

Traditional Chinese medicine extracts are an important topic in medical research. BA has been confirmed to affect the cells' proliferation as well as apoptosis in various tumors.[33-36] Notably, it has been found to have anti-apoptotic effects on bone cells[18,19] such as chondrocytes and osteoblasts. However, no studies have explored the influence of BA on BMSCs.

The HH signaling pathway is important in MSC proliferation and apoptosis.[28,29] SUFU and GLI-1 are downstream effectors of SHH in the HH signal pathway. More specifically, SUFU releases the bound GLI-1 into the nuclei of cells. In the nuclei, it controls the target genes' transcription by binding to their promoters.[37] A growing number of studies have reported that many effector molecules that regulate cell proliferation and apoptosis, such as cyclin-D, cyclin-E, Bcl-2, Bax, and caspase-3, are downstream molecules of the HH signaling pathway.[38-40] We detected molecules associated with the HH signaling pathway in hBMSCs cultured with a high concentration of Dex. Reductions in SHH and GLI-1 expression and increases in SUFU expression indicate inhibition of the HH signaling pathway. Decreases in GLI-1 expression leads to the activation of the mitochondrial apoptotic pathway, which manifests in the increase and decrease, respectively, of Bax and Bcl-2. Increases in cleaved caspase-3 levels results in cell apoptosis. Our findings are consistent with those from previous papers. When we added BA to a cell culture medium containing a high concentration of Dex, the HH signaling pathway was activated, which decreased cell apoptosis. Notably, CP, an inhibitor of the HH signaling pathway, partially weakened the anti-apoptotic effects of BA. Altogether, based on these results, we can reasonably infer that BA ameliorates Dex-induced hBMSC apoptosis by activating the HH signaling pathway.

During the experimental process, we set a concentration gradient for BA. At the cellular level, the proportion of apoptotic cells decreased with increasing BA concentrations. At the molecular level, HH-signaling-pathway-related and apoptosis-related molecules varied with BA concentration. The optimal concentration of BA is worthy of further exploration.

There were some limitations in our study. First, hBMSC apoptosis induced by a high concentration of Dex cannot fully encapsulate the pathological changes in the actual disease process. Second, the metabolism of drugs in the body is a complex process that requires further experimental verification in animals.

In conclusion, this study confirms that BA can block the effect of Dex on hBMSC apoptosis by activating the HH signaling pathway, and this effect is concentration-dependent.

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Conflicts of interest

None.

References

1. Deng YB, Ye WB, Hu ZZ, Yan Y, Wang Y, Takon BF, *et al*. Intravenously administered BMSCs reduce neuronal apoptosis and promote neuronal proliferation through the release of VEGF after stroke in rats. Neurol Res 2010;32:148–156. doi: 10.1179/174313209X414434.
2. Fink T, Rasmussen JG, Emmersen J, Pilgaard L, Fahlman A, Brunberg S, *et al*. Adipose-derived stem cells from the brown bear (Ursus arctos) spontaneously undergo chondrogenic and osteogenic differentiation in vitro. Stem Cell Res 2011;7:89–95. doi: 10.1016/j. scr.2011.03.003.
3. Uccelli A, Moretta L, Pistoia V. Mesenchymal stem cells in health and disease. Nat Rev Immunol 2008;8:726–736. doi: 10.1038/nri2395.
4. Houdek MT, Wyles CC, Packard BD, Terzic A, Behfar A, Sierra RJ. Decreased osteogenic activity of mesenchymal stem cells in patients with corticosteroid-induced osteonecrosis of the femoral head. J Arthroplasty 2016;31:893–898. doi: 10.1016/j.arth.2015.08.017.
5. Nuttall ME, Gimble JM. Is there a therapeutic opportunity to either prevent or treat osteopenic disorders by inhibiting marrow adipogenesis? Bone 2000;27:177–184. doi: 10.1016/s8756-3282(00)00317-3.
6. Hofbauer LC, Rauner M. Minireview: Live and let die: molecular effects of glucocorticoids on bone cells. Mol Endocrinol 2009;23:1525–1531. doi: 10.1210/me.2009-0069.
7. Carcamo-Orive I, Gaztelumendi A, Delgado J, Tejados N, Dorronsoro A, Fernandez-Rueda J, *et al*. Regulation of human bone marrow stromal cell proliferation and differentiation capacity by glucocorti-coid receptor and AP-1 crosstalk. J Bone Miner Res 2010;25:2115–2125. doi: 10.1002/jbmr.120.
8. Rauch A, Seitz S, Baschant U, Schilling AF, Illing A, Stride B, *et al*. Glucocorticoids suppress bone formation by attenuating osteoblast differentiation via the monomeric glucocorticoid receptor. Cell Metab 2010;11:517–531. doi: 10.1016/j.cmet.2010.05.005.
9. Song IH, Caplan AI, Dennis JE. Dexamethasone inhibition of confluence-induced apoptosis in human mesenchymal stem cells. J Orthop Res 2009;27:216–221. doi: 10.1002/jor.20726.
10. Fan Q, Zhan X, Li X, Zhao J, Chen Y. Vanadate inhibits dexamethasone-induced apoptosis of rat bone marrow-derived mesenchymal stem cells. Ann Clin Lab Sci 2015;45:173–180.
11. Li T, Xu Y, Wang Y, Jiang Y. Differential expression profiles of long noncoding RNAs and mRNAs in human bone marrow mesenchymal stem cells after exposure to a high dosage of dexamethasone. Stem Cell Res Ther 2021;12:9. doi: 10.1186/s13287-020-02040-8.
12. Cheng F, Lu Y, Zhong X, Song W, Wang X, Sun X, *et al*. Baicalin's therapeutic time window of neuroprotection during transient focal cerebral ischemia and its antioxidative effects in vitro and in vivo. Evid Based Complement Alternat Med 2013;2013:120261. doi: 10.1155/2013/120261.
13. Cui L, Feng L, Zhang ZH, Jia XB. The anti-inflammation effect of baicalin on experimental colitis through inhibiting TLR4/NF-kappaB pathway activation. Int Immunopharmacol 2014;23:294–303. doi: 10.1016/j.intimp.2014.09.005.
14. Yang JY, Li M, Zhang CL, Liu D. Pharmacological properties of baicalin on liver diseases: a narrative review. Pharmacol Rep 2021;73:1230–1239. doi: 10.1007/s43440-021-00227-1.
15. Checker R, Sharma D, Sandur SK, Khanam S, Poduval TB. Anti-inflammatory effects of plumbagin are mediated by inhibition of NF-kappaB activation in lymphocytes. Int Immunopharmacol 2009;9:949–958. doi: 10.1016/j.intimp.2009.03.022.
16. Xing D, Gao H, Liu Z, Zhao Y, Gong M. Baicalin inhibits inflammatory responses to interleukin-1beta stimulation in human chondrocytes. J Interferon Cytokine Res 2017;37:398–405. doi: 10.1089/jir.2017.0030.
17. Yang X, Zhang Q, Gao Z, Yu C, Zhang L. Baicalin alleviates IL-1beta-induced inflammatory injury via down-regulating miR-126 in chondrocytes. Biomed Pharmacother 2018;99:184–190. doi: 10.1016/j.biopha.2018.01.041.
18. Li Z, Cheng J, Liu J. Baicalin protects human OA chondrocytes against IL-1beta-induced apoptosis and ECM degradation by activating autophagy via MiR-766-3p/AIFM1 axis. Drug Des Devel Ther 2020;14:2645–2655. doi: 10.2147/DDDT.S255823.
19. Cao J, Zhang Y, Wang T, Li B. Endoplasmic reticulum stress is involved in baicalin protection on chondrocytes from patients with osteoarthritis. Dose Response 2018;16:1559325818810636. doi: 10.1177/1559325818810636.
20. Zhu L, Liu YJ, Shen H, Gu PQ, Zhang L. Astragalus and baicalein regulate inflammation of mesenchymal stem cells (MSCs) by the mitogen-activated protein kinase (MAPK)/ERK pathway. Med Sci Monit 2017;23:3209–3216. doi: 10.12659/msm.902441.
21. Qiu L, Chen J, Lin J, Wo D, Chu J, Peng J. Baicalin alleviates H2O2 induced injury of H9c2 cardiomyocytes through suppression of the Wnt/betacatenin signaling pathway. Mol Med Rep 2017;16:9251–9255. doi: 10.3892/mmr.2017.7748.
22. Ingham PW, McMahon AP. Hedgehog signaling in animal development: paradigms and principles. Genes Dev 2001;15:3059–3087. doi: 10.1101/gad.938601.
23. Lee Y, Miller HL, Jensen P, Hernan R, Connelly M, Wetmore C, *et al*. A molecular fingerprint for medulloblastoma. Cancer Res 2003;63:5428–5437.
24. Kinzler KW, Bigner SH, Bigner DD, Trent JM, Law ML, O'Brien SJ, *et al*. Identification of an amplified, highly expressed gene in a human glioma. Science 1987;236:70–73. doi: 10.1126/science.3563490.
25. Katoh Y, Katoh M. Hedgehog target genes: mechanisms of carcinogenesis induced by aberrant hedgehog signaling activation. Curr Mol Med 2009;9:873–886. doi: 10.2174/156652409789105570.
26. Beachy PA, Karhadkar SS, Berman DM. Tissue repair and stem cell renewal in carcinogenesis. Nature 2004;432:324–331. doi: 10.1038/nature03100.
27. Guo Q, Xuan MF, Luo ZB, Wang JX, Han SZ, Ri MH, *et al*. Baicalin improves the in vitro developmental capacity of pig embryos by inhibiting apoptosis, regulating mitochondrial activity and activating sonic hedgehog signaling. Mol Hum Reprod 2019;25:538–549. doi: 10.1093/molehr/gaz036.
28. Hong IS, Kang KS. The effects of Hedgehog on the RNA-binding protein Msi1 in the proliferation and apoptosis of mesenchymal stem cells. PLoS One 2013;8:e56496. doi: 10.1371/journal.pone.0056496.
29. Wang H, Zhao Z, Du J, Wang T, Yang L, Yu H. MiRNA-378 controls cell proliferation in rabbit umbilical cord mesenymal stem cells. Cell Mol Biol (Noisy-le-grand) 2016;62:97–101. doi: 10.14715/cmb/2016.62.12.17.
30. Otsuru S, Hofmann TJ, Olson TS, Dominici M, Horwitz EM. Improved isolation and expansion of bone marrow mesenchymal stromal cells using a novel marrow filter device. Cytotherapy 2013;15:146–153. doi: 10.1016/j.jcyt.2012.10.012.
31. Yeung DK, Griffith JF, Antonio GE, Lee FK, Woo J, Leung PC. Osteoporosis is associated with increased marrow fat content and decreased marrow fat unsaturation: a proton MR spectroscopy study. J Magn Reson Imaging 2005;22:279–285. doi: 10.1002/jmri.20367.
32. Xu Y, Jiang Y, Wang Y, Ren Y, Zhao Z, Wang T, *et al*. LINC00473 regulated apoptosis, proliferation and migration but could not reverse cell cycle arrest of human bone marrow mesenchymal stem cells induced by a high-dosage of dexamethasone. Stem Cell Res 2020;48:101954. doi: 10.1016/j.scr.2020.101954.
33. Sui X, Han X, Chen P, Wu Q, Feng J, Duan T, *et al*. Baicalin induces apoptosis and suppresses the cell cycle progression of lung cancer cells through downregulating Akt/mTOR signaling pathway. Front Mol Biosci 2020;7:602282. doi: 10.3389/fmolb.2020.602282.

34. Singh S, Meena A, Luqman S. Baicalin mediated regulation of key signaling pathways in cancer. Pharmacol Res 2021;164:105387. doi: 10.1016/j.phrs.2020.105387.
35. Huang L, Peng B, Nayak Y, Wang C, Si F, Liu X, *et al*. Baicalein and baicalin promote melanoma apoptosis and senescence via metabolic inhibition. Front Cell Dev Biol 2020;8:836. doi: 10.3389/fcell.2020.00836.
36. Yang B, Bai H, Sa Y, Zhu P, Liu P. Inhibiting EMT, stemness and cell cycle involved in baicalin-induced growth inhibition and apoptosis in colorectal cancer cells. J Cancer 2020;11:2303–2317. doi: 10.7150/jca.37242.
37. Rimkus TK, Carpenter RL, Qasem S, Chan M, Lo HW. Targeting the sonic hedgehog signaling pathway: review of smoothened and GLI inhibitors. Cancers (Basel) 2016;8:22. doi: 10.3390/cancers8020022.
38. Zhao DW, Hou YS, Sun FB, Han B, Li SJ. Effects of miR-132 on proliferation and apoptosis of pancreatic cancer cells via Hedgehog signaling pathway. Eur Rev Med Pharmacol Sci 2019;23:1978–1985. doi: 10.26355/eurrev_201903_17236.
39. Kim BR, Jeong YA, Na YJ, Park SH, Jo MJ, Kim JL, *et al*. Genipin suppresses colorectal cancer cells by inhibiting the Sonic Hedgehog pathway. Oncotarget 2017;8:101952–101964. doi: 10.18632/onco-target.21882.
40. Lin J, Wei L, Shen A, Cai Q, Xu W, Li H, *et al*. Hedyotis diffusa Willd extract suppresses Sonic hedgehog signaling leading to the inhibition of colorectal cancer angiogenesis. Int J Oncol 2013;42:651–656. doi: 10.3892/ijo.2012.1753.

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