Dexamethasone promotes the endoplasmic reticulum stress response of bone marrow mesenchymal stem cells by activating the PERK-Nrf2 signaling pathway

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Abstract

The pathogenesis of steroid-induced avascular necrosis of femoral head (SANFH) is complex, and there is a lack of effective early prevention method. The aim of the present study was to evaluate the effect of dexamethasone (DEX) on the biological behavior of bone marrow mesenchymal stem cells (BMSCs) and to explore the possibility of DEX in the clinical treatment of SANFH. The effect of DEX on the proliferation of BMSCs was evaluated by Counting Kit-8 assay, western blot assay, and enzyme-linked immunosorbent assay. Flow cytometry and western blot assay were performed to detect the effect of DEX on the apoptosis of BMSCs. Quantitative real-time PCR and western blot assay were performed to detect the effect of DEX on the expression of endoplasmic reticulum stress (ERS)-related genes. Immunoblotting analysis was conducted for detecting the nuclear-cytoplasmic distribution of Nrf2. DEX could significantly inhibit the proliferation of BMSCs and promote apoptosis of BMSCs. DEX could increase the expression of PERK, ATF6, and IRE1α, and induce nuclear translocation of Nrf2. The addition of ML385 could reverse the effect of DEX on BMSCs. DEX could activate the PERK-Nrf2 pathway to promote ERS and finally affect the cell proliferation and apoptosis of BMSCs.

KEYWORDS
bone marrow mesenchymal stem cells, dexamethasone, endoplasmic reticulum stress, steroid-induced avascular necrosis of femoral head
Steroid-induced avascular necrosis of femoral head (SANFH) is systemic multiple osteonecrosis caused by the massive use of glucocorticoids. The main symptoms of SANFH are hip pain, joint stiffness, and limited mobility. If there is no timely intervention, SANFH will cause the collapse of the articular surface and osteoarthritis and eventually lead to the disability and deformity of patients. Currently, the pathogenesis of SANFH is still not clear, and there is no effective early prevention method. Bone marrow mesenchymal stem cells (BMSCs) are a type of stromal stem cells that can participate in the regeneration and repair of various tissues. Studies have shown that under the action of glucocorticoids, BMSCs can differentiate into adipocytes and cause cell proliferation and hypertrophy, resulting in increased local intraosseous pressure, blood circulation disorders, and bone ischemic necrosis.

The endoplasmic reticulum (ER) acts as an organelle for protein synthesis folding, lipid synthesis, and cell membrane structure in cells. ER widely participates in the regulation of cell stress and apoptosis, and plays an important role in maintaining cell homeostasis. When cells are under conditions of ischemia/hypoxia, oxidative stress, viral infection, nutritional deficiencies and increased stress, ER stress (ERS) will occur, resulting in a large number of unfolded proteins accumulating in the ER and unfolded protein response (UPR), to restore the homeostasis of the ER and protect the cells. However, when ERS response is too strong and the duration is too long, it will lead to physiological dysfunction, imbalance of homeostasis, and apoptosis.

In mammals, the ER maintains the balance of the intracellular environment by regulating protein synthesis and folding process mainly by activating three signal pathways of three ER transmembrane ERS sensors (1) inositol-requiring enzyme 1a (IRE1a); (2) the double-stranded RNA-activated protein kinase-like eukaryotic initiation factor 2α kinase (PERK); (3) activating transcription factor 6 (ATF6). The UPR sensors are all maintained in an inactive state through interaction between their ER luminal domains with the protein chaperone, immunoglobulin heavy-chain binding protein (BiP; also known as GRP78 and HSPA5). When unfolded/misfolded proteins accumulate in the ER upon ERS, they bind to and sequester BiP, thereby promoting BiP dissociation and initiating downstream signaling. The PERK pathway is the immediate early-response pathway among the three ERS pathways. During activation, PERK phosphorylates eukaryotic initiator factor 2e (eIF2α) to promote activating transcription factor 4 (ATF4) translation and then upregulates light chain 3 (LC3), which participates in elongation and maturation of autophagosomes. ATF4 also activates the transcription of the C/EBP homologous protein (CHOP), which is essential for the apoptotic response to ERS.

Nuclear factor E2-related factor 2 (Nrf2), a basic leucine zipper (bZIP) protein, was previously thought to protect against oxidative stress. However, recently, it has been found that Nrf2 is also an important downstream factor of PERK. PERK activates Nrf2, which then translocates into the nucleus, thereby attenuating protein translation and inducing genes controlling redox homeostasis.

Dexamethasone, a synthetic glucocorticoids hormone, has been identified to inhibit the synthesis of fibronectin and collagen, and to activate collagenase synthesis. Current studies have found that high doses of dexamethasone (DEX) may affect the lipid metabolism of BMSCs. The ER is the major organelle involved in lipid metabolism, as the ER contains many relevant enzymes for lipid metabolism. Several reports indicate that the PERK-eIF2α pathway regulates lipogenesis and hepatic steatosis. For example, PERK and eIF2α phosphorylation are induced by antipsychotic drugs, resulting in increased lipid accumulation in hepatocytes by the activation of SREBP-1c and SREBP-2. Mice with compromised eIF2α phosphorylation by the overexpression of GADD34 in the liver, displayed reduced high-fat diet (HFD)-induced hepatosteatosis. ATF4, the downstream gene of the PERK/eIF2α pathway, was also reported to be involved in lipid metabolism. Based on this, we speculated that DEX might affect the lipid metabolism of BMSCs by regulating the ERS response, thereby regulating proliferation and apoptosis, and ultimately affecting the progress of SANFH.

In the present study, we explored the effect of DEX on the proliferation and apoptosis of BMSC cells and the expression of ERS-related proteins in BMSC cells, and initially clarified the molecular mechanism. Our findings might be valuable for DEX clinical application in the treatment of SANFH.

2 | MATERIALS AND METHODS

2.1 | BMSCs culture

Primary human BMSCs were purchased from Otwo Biotech (ShenZhen) Inc. BMSCs were cultured in Dulbecco’s modified eagle media (DMEM)/1% penicillin/streptomycin/10% exosome-depleted fetal bovine serum. Cell cultures were maintained at 37°C in 5% CO2 with medium changed every 3 days and cells passaged with 0.05% trypsin/EDTA.

Thapsigargin (T135258, Aladdin, ≥95%) was a specific inducer of ERS. In this study, Thapsigargin with a final concentration of 100 nM was added to the medium to pre-culture for 48 h to induce ERS as a positive control.

ML385 (an Nrf2 inhibitor, purchased from AbMole, M8692) was used to further verify whether DEX activated ERS by the PERK-Nrf2 signaling pathway and the method was followed as previously described. ML385 was dissolved in PBS with 5% DMSO and the concentration was set as 10 μM.

2.2 | Enzyme-linked immunosorbent assay (ELISA)

BMSCs were seeded in 96-well plates in triplicate at a density of 1 × 10^5 cells/well and cultured for 72 h at 37°C with 5% CO2 in a humidified incubator. A Quantikine ELISA Kit Human IGF1 (R&D Systems) was used to further verify whether DEX activated ERS by the PERK-Nrf2 signaling pathway and the method was followed as previously described.
Systems) was used in accordance with the manufacturer’s instructions to measure IGF1 level in the cell culture supernatant. The amounts of antigen in the controls and samples were calculated by interpolation from a standard curve.

### 2.3 Cell proliferation assay

BMSCs were seeded into the culture medium containing 10% FBS on a 96-well plate at a cell density of $1 \times 10^5$ cells/well. Cells were incubated with the indicated concentration of DEX for 24 h and then counted using a Cell Counting Kit-8 (CCK-8) (Dojindo). Absorbance was measured with a microplate reader at a wavelength of 490 nm.

### 2.4 Flow cytometry

After treatment with DEX (CSR-CK001, Cosmo Bio), BMSCs were fluorescently labeled using an Annexin V-FITC apoptosis detection kit (Roche Applied Science) in accordance with the manufacturer’s instructions. Briefly, BMSC pellets were collected and washed twice with cold PBS and gently resuspended in 500 μl of a binding buffer containing 5 μl of Annexin V-FITC and 5 μl of PI for 15 min at room temperature. Then, the samples were analyzed using flow cytometry (BD Biosciences).

### 2.5 Western blotting

BMSCs were washed with cold phosphate-buffered saline and lysed in 2X sodium dodecyl sulfate (SDS) sample buffer (100 mM Tris-HCl (pH 6.8), 10 mM EDTA, 4% SDS, and 10% glycerol) for 1 h at 4°C, respectively. After centrifugation (12,000 g for 15 min), the protein content was measured with an enhanced BCA protein assay kit (Beyotime). Equal amounts (30 μg) of protein in each lane were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane (Millipore). For the detection of protein expression, the following antibodies were used: PERK (CST, 3192, 1:1000), ATF6 (Cosmo Bio, AM-73-500-B, 1:1000), TFAR19 (Abcam, ab83958, 1:1000), IGF1 (Cosmo Bio, KM2076, 1:1000), IRE1α (Abcam, ab146176, 1:1000), NRF2 (Abcam, ab62352, 1:1000), β-catenin (CST 8480, 1:1000). Anti-rabbit (111-035-003), and anti-mouse (115-035-003) HRP-conjugated secondary antibodies were purchased from Jackson ImmunoResearch and the signal was visualized using western blotting luminol reagent (Santacruz, sc-2048).

### 2.6 Quantitative real-time PCR (qRT-PCR) validation

Total RNA was extracted from tissues using TRIzol Reagent (Invitrogen). Total RNA was reverse-transcribed using ImProm II Reverse Transcriptase (Promega) according to the manufacturer’s protocol. Real-time PCR was performed using an SYBR Green PCR kit (Ta Ka Ra) according to the manufacturer’s protocol. The relative expression fold change was calculated using the $2^{-\Delta\Delta Ct}$ method and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control. All the experiments were performed in the same condition for at least three individual repeats. The following sequence-specific primers were synthesized:

- GAPDH sense: 5’-GGTGAAGTGCGGAGGTCAC-3’;
- GAPDH antisense: 5’-CAAAGTTGCTAGGATGACC-3’;
- IRE1α sense: 5’-GAAATTCATGGCGGGCAGGCGCTGCTGCTG-3’;
- IRE1α antisense: 5’-AAGCTTGAGGGCGCTCGACTGGG-3’;
- PERK sense: 5’-TCATCCAGCTTAGCAAAACC-3’;
- PERK antisense: 5’-ATGCTTTCCAGGCTTGGTC-3’;
- ATF6 sense: 5’-CTTCCTCAGGGTCTCCATC-3’;
- ATF6 antisense: 5’-CAACTCCAGGAAACGTGCT-3’.

### 2.7 Nrf2 transcription factor assay

The nuclear extracts were prepared from treated cells for the Nrf2 transcriptional activity analysis using a commercialized kit according to the manufacturer’s instruction (Abcam).

### 2.8 Statistical analysis

All results represent the mean ± standard deviation from three independent experiments. Student’s t-test was used to evaluate the differences between two groups using SPSS 13.0 software (SPSS, Inc.). Two-way ANOVA was performed to evaluate the difference in multi groups using GraphPad 6.0. Significant significance was set at $p < .05$.

### 3 RESULTS

#### 3.1 DEX inhibits the proliferation of BMSCs

To determine whether DEX affected the proliferation of BMSCs, we first performed a CCK8 assay to detect the effect of different concentrations of DEX on BMSCs proliferation. The concentration of DEX was set as $10^{-9}$ mol/L, $10^{-8}$ mol/L, $10^{-7}$ mol/L, and $10^{-6}$ mol/L, and the medium without DEX was set as control. As shown in Figure 1A, with the increase in the concentration of DEX and the continuation of the cultivation time, the cell activity of BMSCs gradually decreased. Then western blot assay was conducted to determine the expression of cell growth factor IGF1. The results showed that the expression of IGF1 was gradually decreased with the increase of the concentration of DEX, which indicated that DEX could inhibit the proliferation of BMSCs (Figure 1B). Furthermore, ELISA was conducted to quantitatively analyze the secretion of IGF1 from
BMSCs. As shown in Figure 1C, the amount of IGF1 secretion gradually increased with the time prolonging and stabilized from the 20th day at the concentration of DEX ($10^{-7}$ mol/L). All the above data suggested that DEX could inhibit the proliferation of BMSCs with the increase in the concentration.

3.2 DEX promotes the apoptosis of BMSCs.

We next studied the effect of DEX on the apoptosis of BMSCs. The results of flow cytometry analyses showed that, with the increase in the concentration of DEX, the apoptosis percentage of BMSCs gradually increased (Figure 2A and B). Also, the expression of apoptosis-related protein TEAR19 was increased with the increase in the concentration of DEX via western blotting (Figure 2C). These findings indicated that DEX could promote the apoptosis of BMSCs.

3.3 DEX activates ERS of BMSCs

To further study the mechanism of DEX regulating the cell biological behavior of BMSCs, we detected the expression of three well-known ERS-related factors (PERK, IRE1a, and ATF6)
after BMSCs were treated with DEX. The result of qRT-PCR (Figure 3A) showed that the mRNA expression of ATF6, PERK, and IRE1α was significantly increased when the concentration of DEX increased ($p < .01$, $p < .001$). Also, a similar result was obtained from the western blot assay, as showed by an obvious elevation of ATF6, p-PERK, PERK, and p-IRE1α/IRE1α protein expression ($p < .001$) (Figure 3B). Both qRT-PCR and western blot assays suggested that DEX treatment may activate the ERS of BMSCs by regulating the ERS-related pathways, including ATF6, PERK, and IRE1α signaling pathways.

### 3.4 DEX activates ERS of BMSCs by the PERK-Nrf2 pathway

To further confirm the potential molecular mechanism of DEX-induced ERS activation, we then analyzed the nucleus–cytoplasm distribution of Nrf2 proteins in DEX-treated cells. As shown in Figure 4A, DEX treatment significantly elevated the phosphorylated PERK and phosphorylated Nrf2 levels and induced nuclear translocation of Nrf2 proteins. Considering that when the concentration of DEX was increased, the expression of PERK increased most significantly, ML385 (an Nrf2 inhibitor) was used to further verify whether DEX activated ERS by the PERK–Nrf2 signaling pathway. In this process, Thapsigargin was used to induce ERS. As shown in Figure 4B, DEX significantly promoted the phosphorylation of PERK and Nrf2 proteins, induced the increase of TEAR19 and the decrease of IGF1. Thapsigargin, an ERS-specific inducer, had similar effects on these proteins as DEX and was more severe. Combining ML385 on the basis of DEX significantly reduced DEX’s promotion of p-PERK, p-NRF2, and TEAR19, as well as its inhibitory effect on IGF1. This suggested that the use of ML385 might partially alleviate ERS caused by DEX. In Figure 4C, it was shown that ML385 not only promoted cell viability, but also rescued the
suppression effect of DEX on cell viability \( (p < .01) \). Moreover, the addition of ML385 also weakened the promoting effect of DEX on cell apoptosis \( (p < .01) \) (Figure 4D). These data demonstrated that inhibiting PERK-Nrf2 reduced the inhibition of proliferation and the promotion of apoptosis by DEX. All above revealed that DEX affected the cell behavior of BMSCs by activating ERS by the PERK-Nrf2 pathway.

4 | DISCUSSION

Glucocorticoid is a steroid hormone secreted by the human adrenal cortex. It can be used in conjunction with antibiotics or anti-inflammatory drugs to treat certain conditions, such as severe acute respiratory syndrome and sepsis. Glucocorticoids are also involved in regulating the biosynthesis and metabolism of sugar, fat, and protein, and inflammatory response, which have a good therapeutic effect on many diseases especially allergic, infectious, and autoimmune diseases, such as rheumatoid arthritis, asthma, rheumatic polyarthritis, systemic lupus erythematosus, chronic obstructive pulmonary disease, and organ transplantation. In 1953, Pietrogrande et al. reported the first case of femoral head necrosis after glucocorticoid application. In recent years, due to the widespread use of glucocorticoids, the prevalence of SANFH has been rising, accounting for about 51% of non-traumatic femoral head necrosis, and showing the trend of younger people. A large number of studies have shown that the occurrence of SANFH is related to many factors. At present, its pathogenesis and pathological process have not been clarified, but many hypotheses have been proposed, mainly including two theories: 1. necrosis pathogenesis theory, including fat metabolism disorders, osteoporosis, increased intrasosseous pressure, intravascular coagulation, etc. 2. Theory of apoptosis pathogenesis, including the effects of hormone metabolism, bone marrow stromal stem cell fat differentiation, gene polymorphism, etc. Due to the difficulty in repairing osteonecrosis tissue and the need for some patients to continue to use glucocorticoids for a long time, SANFH has caused a very high disability rate, making SANFH widely concerned in the medical community. The development of the disease may cause irreversible damage to the femoral head, seriously affecting the patient’s quality of life, and no treatment can completely reverse the progress of the disease. Therefore, studying the pathogenesis of SANFH is of great significance for prevention, diagnosis, and treatment.

Studies have shown that under the action of glucocorticoids, BMSCs can differentiate into adipocytes and cause cell proliferation and hypertrophy, resulting in bone ischemic necrosis, and high doses of DEX could affect the lipid metabolism of BMSCs. In the present study, we proved that DEX could inhibit the proliferation and promote the apoptosis of BMSCs, and this effect was increased in a dose-independent manner. Our results were in accordance with the previous studies, which found that DEX could both inhibit the proliferation and promote the apoptosis of BMSCs. Abnormal ER function will cause ERS. Early ERS response is mediated by three transmembrane receptors PERK, ATF6, and IRE1. In normal cells, PERK, ATF6, and IRE1 bind to the molecular chaperone GRP78 in the ER cavity to maintain the non-activated state. When stress occurs, they dissociate from three receptors, resulting in receptor activation and ERS. Early ERS is UPR, a pro-survival response, which can help reduce the accumulation of unfolded proteins in the ER and restore the function of the ER. But when UPR is not enough to protect cell survival, ER will be used as the origin of apoptosis signal to induce cell apoptosis, including the expression of transcription factor CHOP, Procaspase-12 shear reaction, and activation of JNK pathway through IRE1. Here, we have shown that DEX could activate ERS of BMSCs (Figure 3). Among PERK, ATF6, and IRE1, the expression of PERK has the most significant change with the change in DEX concentration. Therefore, we mainly explored the potential molecular mechanism of the PERK pathway involving DEX-mediated BMSCs proliferation and apoptosis in the following study.

PERK is well known to have transcriptional effects via the transcription factor Nrf2, a pro-survival transcription factor that moves from the cytoplasm to the nucleus upon phosphorylated by PERK. Previous research has reported that Nrf2 was also an important downstream factor of PERK. PERK could activate Nrf2 to attenuate protein translation and induce genes controlling redox homeostasis, which would induce apoptosis. To further identify the effect of DEX on the expression of PERK and Nrf2, we detected the expression change in PERK and Nrf2 with different concentrations of DEX. We found that DEX could increase the phosphorylation level of PERK and Nrf2, and DEX could also induce nuclear translocation of Nrf2 proteins. When ERS is excessive, ER-related apoptosis is initiated. To further explore whether DEX could induce the apoptosis of BMSCs by the PERK-Nrf2 pathway, ML385 was used to inhibit the expression of Nrf2. The results of the rescue experiment have shown that the addition of ML385 could reverse the effect of DEX on the cell behaviors of BMSCs, indicating that DEX promotes apoptosis and suppress the cell viability of BMSCs by regulating the PERK-Nrf2 pathway. However, the study shows that PERK can promote Nrf2 through ATF4, thereby controlling ERS and alleviating cell damage caused by reactive oxygen species. This study reveals another regulatory mechanism by which PERK promotes the expression of Nrf2 protein.

This suggests that there are many ways of regulation between PERK and Nrf2. In the process of BMSCs ERS caused by DEX, the molecular mechanism of PERK-Nrf2 needs to be further studied.

5 | CONCLUSION

In the present study, we explored the effect of DEX on the cell behavior of BMSCs. The results showed that DEX could promote apoptosis and inhibit the cell viability of BMSCs, which also plays an important role in regulating ERS of BMSCs. Further in-depth mechanistic studies revealed that DEX could activate the ERS of BMSCs and affect the cell behavior of BMSCs by activating the PERK-Nrf2 pathway. Our research may provide a theoretical basis for the use of DEX in the clinical treatment of SANFH.
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None.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

AUTHORS’ CONTRIBUTIONS

Suoli Cheng and Jiandang Shi designed the research. Suoli Cheng wrote the manuscript with contributions from all authors. All authors participated in part of the experiment. Xueqing Liu, Fan Gong, Xiaolin Ding, and Xuebing Zhou are responsible for data acquisition and analysis. Cuiyun Liu is responsible for statistical analysis. Fei Zhao and Xiaoliang Li are responsible for literature search. Jiandang Shi reviewed and modified the article. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the local ethics committee. Informed consent was obtained from all participating subjects.

DATA AVAILABILITY STATEMENT

The data sets analyzed during the current study are available from the corresponding author on reasonable request.

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