Research Article

Human Mesenchymal Stem Cells-Derived Exosome Mimetic Vesicles Regulation of the MAPK Pathway and ROS Levels Inhibits Glucocorticoid-Induced Apoptosis in Osteoblasts


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1. Introduction

Nontraumatic osteonecrosis of the femoral head (NONFH) is the ischemic necrosis or aseptic necrosis of the femoral head due to various nontraumatic factors [1, 2]. Among them, steroid hormones overuse is the most common nontraumatic cause of osteonecrosis of the femoral head (ONFH) [3]. Epidemiological studies in East Asia showed that nontraumatic ONFH was directly associated with steroid hormones in 47.4% of cases [4]. Steroid hormones induced ONFH has always been one of the most serious orthopedic diseases. There are many possible pathogenesis...
and risk factors, among which oxidative stress disorder may be one of the most common factors involved, and the oxidative stress caused by long-term use of glucocorticoids can weaken the repair capacity of bone tissue [5, 6]. Oxidative stress refers to a state where the balance between the production of prooxidants (free radicals or reactive oxygen species (ROS)) and the elimination of antioxidants is disturbed in cells; in recent years, a number of studies have shown that oxidative stress can cause apoptosis through mitochondrial-dependent pathway [7]. Previous studies generally believe that ROS accumulation can induce osteonecrosis in ONFH [8]. Increasing evidence suggests a critical role for osteogenic cell apoptosis in steroid hormones induced ONFH [9]. While ROS is involved in the steroid hormones induced apoptosis of the osteogenic cells [5].

Treatment of steroid hormones induced ONFH remains an orthopedic problem, with hip replacement being the only treatment option at the end of phase 1, affecting over 10,000 new patients each year in the United States, accounting for 10% of total hip replacement (THAs) [10]. Considering the serious consequences and economic costs caused by ONFH, it is necessary to find new treatments. Recently, stem cell transplantation has been used for the treatment of early hormonal ONFH [11]. However, studies have found that the transplanted stem cells undergo a large amount of stress-dependent pathway [7]. Previous studies generally believe oxidative stress can cause apoptosis through mitochondria-dependent pathway [7]. Previous studies generally believe that ROS accumulation can induce osteonecrosis in ONFH [8]. Increasing evidence suggests a critical role for osteogenic cell apoptosis by regulating ROS levels, in order to provide potentially viable therapeutic measures for hormone-induced ONFH.

2. Materials and Methods

2.1. Cell Culture. Human umbilical cord MSCs (HucMSCs) (cat. no. PCS-500-010) were provided by Chongqing Stem Cell Biotechnology R&D Base, Chongqing, China. HucMSCs were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) and 1% P/S (streptomycin double antibody). Osteoblast MC3T3-E1 was purchased from the official website of the Shanghai Cell Bank, and were cultured in pefc medium (Procell, stock number CM-0378) which contains α-MEM (PM150421), 10% FBS (164210-50), and 1% P/S (PB180120). All cells were incubated in 37°C, 5% CO₂ in air.

2.2. Preparation of hucMSC-EMVs. HucMSCs were collected, and resuspended in PBS, then extruded using a micro extruder with polycarbonate membrane filters with various pore sizes (10, 5, and 1 µm) (Avanti Polar Lipids). The extruded samples were collected for ultracentrifugation at 100,000 × g for 1 hr at 4°C, and finally the precipitates were collected, resuspended using PBS, and filtered through 0.22 µm filters. The specific step of preparing hucMSC-EMVs is the same as the anterior [16].

2.3. Characterization of hucMSC-EMVs. We used transmission electron microscopy to observe the morphology of hucMSC-EMVs, used nanoparticle tracking analysis to detect the size of hucMSC-EMVs, and used western blot to detect the surface markers of exosomes (CD63, Alix, and TSG101). The specific method was introduced in the previous work [16].

2.4. Cell Viability Was Determined by Cell Counting Kit-8 (CCK-8). Three thousand MC3T3-E1 cells/well were plated to 96-well plates, and the cells were routine cultured for 24 hr until fully adherent, and then were treated with different concentrations of DEX added (0, 0.05, 0.1, 0.25, 0.5, 0.75, 1.2, and 4 µM) for 48 hr. The medium was then discarded, 10 µl CCK-8 working solution and 90 µl fresh medium were added, and incubation was continued for 2 hr at 37°C, followed measuring absorbance at 450 nm using a microplate reader. After the intervention concentration of DEX was determined, MC3T3-E1 cells were also added to 96-well plates, and the cells were routine cultured for 24 hr until fully adherent, and then different concentrations of hucMSC-EMVs (0, 6.25, 12.5, 25, 25, 50, 75, 100, 200, and 400 µg/ml) were added, then DEX was added 6 hr later, after 48 hr of incubation, CCK-8 working solution was added as described previously, and the absorbance was measured after 2 hr. On the issue of the timing and dose of DEX and hucMSC-EMVs, we referred to the previous studies and selected a significantly different concentration as a subsequent intervention dose based on the results of CCK-8 assay [19–21].

2.5. RNA Sequencing and Transcriptomic Analysis. To elucidate the specific mechanism of dexamethasone-causing
Figure 1: Continued.
apoptosis in MC3T3-E1 cells, we sent DEX-treated MC3T3-E1 cells and control MC3T3-E1 cells samples to Lianchuan Biological Co., Ltd. for RNA sequencing, and we further screened differentially expressed genes between DEX-treated and control cells, followed by KEGG enrichment analysis of differentially expressed genes. The specific steps are mentioned in the previous literature [22].

2.6. hucMSC-EMVs Endocytosis Experiments. hucMSC-EMVs was labeled with the membrane dye PKH26 according to the manufacturer’s instructions, using 800 µl EMVs (1 mg/ml), added with 1,000 µl DilueneC, vortexed for 1 min, added 5 µl PKH26 solution, incubated at 37°C for 15 min, stopped staining, and used 100,000 g. 4°C for 70 min. The precipitates were resuspended with the culture medium. MC3T3-E1 cells were seeded in 24-well plates containing cell sheets and then added PKH26-labeled EMVs at a concentration of 100 µg/ml. After 0, 12, 24, and 48 hr incubation, cells were washed with PBS, fixed in 4% paraformaldehyde, stained with DAPI, and photographed under a fluorescence microscope (Nikon, K10587, Japan).

2.7. hucMSC-EMVs and N-Acetyl L-Cysteine (NAC) Treatment. NAC was dissolved in PBS, and 100 µg/ml EMVs [21], and 5 mM NAC were selected to treat MC3T3-E1 cells based on our previous experimental results [23].

2.8. Flow Cyt Apoptosis. Apoptotic cell death was measured using an Apoptosis Detection Kit (BD, SanDiego, CA). In brief, DEX, EMV, and NAC were intervened in MC3T3E1 cells for 48 hr. After the cells were digested with trypsin without EDTA and washed with PBS, they were resuspended in binding buffer and stained with annexin V fluorescein isothiocyanate (FITC) and PI. The mixture was incubated at room temperature for 15 min, and cells were analyzed by flow cytometry. Specific steps were performed as described in the by Puppel et al. [5]. Apoptotic cells were counted by flow cytometry (BD Biosciences, Franklin Lake, NJ), and the annexin V/PI ratio reflects the percentage of apoptosis.

2.9. Western Blot. Cellular proteins were extracted using radioimmunoprecipitated RIPA (HY-K1001, MCE) reagent supplemented with a 1% protease inhibitor cocktail (HY-K01010, MCE). Protein concentration was determined by using a BCA Assay Kit. The method of western blot is the same as our previous work [16], image acquisition and densitometry analysis were performed using ImageLab (version 6.0.0, USA). The antibody information used is placed in Table S1.

3. Results

3.1. DEX-Induced Apoptosis of MC3T3-E1 and Increased ROS Level. We first intervened the osteoblasts by dexamethasone with different concentration gradient, looking for the dose causing the altered cell viability as the best intervention dose, and we found that the osteoblasts viability decreased significantly at DEX 1 and 2 µM (Figure 1(a)). We found by flow cytometry that the level of apoptosis in osteoblasts increased significantly after DEX intervention (Figures 1(b) and 1(c)), and the western blot results showed that the expression of proapoptotic protein B-cell lymphoma 2- (Bcl 2-) associated X (Bax) increased significantly and the expression

![Figure 1: DEX induced apoptosis in MC3T3-E1 cells. (a) Cell viability of MC3T3-E1 after intervention with different concentrations of dexamethasone. (b and c) Level of apoptosis in MC3T3-E1 cells after DEX intervention by flow cytometry. (d and e) Expression of apoptosis-related proteins Bax and Bcl 2 after DEX intervention. (f) ROS generation level of MC3T3-E1 cells after DEX intervention (scale bar: 100 µm). (g) and (h) HO-1 and Nrf 2 expression after DEX intervention by western blot. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001 compared with control group.](image-url)
FIGURE 2: Continued.
of antiapoptotic protein Bcl-2 decreased significantly after DEX intervention (Figures 1(d) and 1(e)). We further examined the ROS level in MC3T3-E1 after DEX intervention, and found that the ROS level was significantly increased after DEX intervention (Figure 1(f)), and the western blot results showed that the expression level of oxidative stress-related proteins HO-1 and nuclear factor erythroid-derived 2-like 2 (Nrf 2) was significantly increased (Figures 1(g) and 1(h)).

3.2. Transcriptomic Analysis of MC3T3-E1 Cells from Control and DEX-Treated Mice. After transcriptomic analysis of MC3T3-E1 cells after DEX intervention and control group, the heatmap of differential genes showed good clustering of the two groups (Figure 2(a)). The volcano plot showed that 1,274 differential genes were found between the two groups, of which 643 were upregulated and 631 were downregulated (Figure 2(b)). KEGG enrichment analysis of the differential genes showed significant enrichment to the MAPK-signaling pathway (Figure 2(c)).

3.3. DEX Leads to the Activation of the MAPK-Signaling Pathway in MC3T3-E1 Cells. According to the results of the KEGG enrichment analysis, we considered that the osteoblast apoptosis induced by DEX is likely due to the activation of the MAPK-signaling pathway. Therefore, we examined the changes of the key proteins p-JNK, p-ERK, and p-p38 in the MAPK-signaling pathway in osteoblasts after DEX intervention, and found that the MAPK-signaling pathway was significantly activated (Figures 2(d) and 2(e)).

3.4. Characterization of hucMSC-EMVs. We successfully extracted hucMSC-EMVs by continuously squeezing cells, and transmission electron microscopy showed that MSCs’ vesicles were round particles with double membrane structure (Figure 3(a)), with a diameter concentrated between 100 and 200 nm (Figure 3(b)). The results of western blot showed that the exosome marker proteins ALIX, TSG101, and CD63 are equally enriched in the hucMSC-EMVs (Figure 3(c)).

3.5. Endocytosed of hucMSC-EMVs in MC3T3-E1 Cells. We examined whether MC3T3-E1 were capable of endocytosing hucMSC-EMVs. The results showed that MC3T3-E1 could absorb hucMSC-EMVs; hucMSC-EMVs were not endocytosed into the cytoplasm at 0 hr, but they were endocytosed into the cytoplasm at 12, 24, and 48 hr, and EMVs endocytosis increased over time and increased along the nuclear membrane at 24 and 48 hr (Figure 4).

3.6. hucMSC-EMVs and NAC Attenuate Apoptosis of MC3T3-E1 Cells Caused by DEX. We determined the optimal dose of hucMSC-EMVs to repair DEX-induced osteoblast injury using CCK-8, which showed that osteoblast viability increased after the EMVs dose above 25 µg/ml compared with the DEX treatment group, and the difference was more significant after 100 µg/ml EMVs intervention (Figure 5(a)). Therefore, the 100 µg/ml EMVs intervention was selected for all the subsequent experiments. We further measured the apoptosis level of MC3T3-E1 by flow cytometry after EMVs and NAC intervention, and found that both EMVs and NAC significantly inhibited DEX-induced osteoblast apoptosis (Figures 5(b) and 5(c)). The results of western blot showed that Bax level significantly decreased after EMVs and NAC intervention compared with DEX alone group, while Bcl 2 level significantly increased (Figures 5(d) and 5(e)).

3.7. hucMSC-EMVs and NAC Can Inhibit DEX-Induced Elevation of ROS Levels and MAPK-Signaling Pathway Activation in MC3T3-E1 Cells. To verify whether hucMSC-EMVs affected DEX-induced osteoblast apoptosis by inhibiting ROS level and MAPK signaling, we further examined the ROS levels after EMVs and NAC intervention, and found that EMVs could inhibit the DEX-induced ROS levels with the same effect as NAC (Figure 6(a)). The results of western blot showed that adding EMVs and NAC significantly inhibited the increase of HO-1 and Nrf 2 levels caused by DEX intervention.
In addition, EMVs ((Figure 7(a)) and NAC (Figure 7(b)) significantly inhibited the DEX-induced activation of the MAPK-signaling pathway.

4. Discussion

Our study found that DEX induces apoptosis in MC3T3-E1. Further combination of transcriptomic analysis found that oxidative stress and MAPK signaling play a key role in it, and verified that DEX leads to increased ROS levels, in addition, DEX leads to activation of MAPK-signaling pathway. We also found that hucMSC-EMVs intervention significantly reduced DEX-induced osteoblast apoptosis and reducing ROS levels as well as inhibiting the MAPK-signaling pathway, with the same effect as the ROS inhibitor NAC. In brief, EMV may contribute to steroid-induced necrotic repair of the femoral head by regulating ROS levels and the MAPK-signaling pathway.
Epidemiological data suggest that the occurrence and development of ONFH are closely related to steroid hormones [25]. Long-term use or high doses of steroid hormones may cause osteonecrosis [26]. Osteoblasts play a key role in the osteogenesis process and in the prevention of osteonecrosis [27]. As a widely used glucocorticoid, DEX can promote the apoptosis of osteoblasts [28, 29]. ROS accumulation is associated with several diseases, ROS-mediated apoptosis was also demonstrated in AlCl₃-induced dysfunction of MC3T3-E1 cells as studies by Liu et al. [30]. ROS involvement was also observed in LPS-induced apoptosis of the lung epithelial cells [31]. Several studies confirm that DEX may promote excessive generation of ROS as well as disruption of the antioxidative stress system, thereby inducing osteoblast death [32–34]. Bai et al. [6] showed that ROS was significantly increased in the DEX-induced osteoblast apoptosis, and that the ROS scavenger N-acetylcysteine (NAC) significantly reduced the DEX-induced ROS increase, thereby inhibiting ROS-induced osteoblast apoptosis. Our study also confirmed that DEX can promote the ROS accumulation and induce apoptosis in the osteoblasts.

DEX can induce ROS accumulation and promote osteoblast apoptosis has been confirmed in many studies, but the mechanism of DEX-induced osteoblast apoptosis is still unclear, so we further conducted transcriptome sequencing of osteoblasts and control osteoblasts after DEX intervention and screened for related pathways. We successfully enriched to the MAPK-signaling pathway by two sets of differentially expressed genes. MAP kinase (MAPK), a new serine/threonine kinase, catalyzes the phosphorylation of microtubule-associated protein 2 (MAP-2) in insulin-treated 3T3-L1 adipocytes, and extracellular signal-regulated kinase 1 (ERK 1), a cloned product of MAP kinase [35, 36]. Stress-activated JNKs and p38 MAPKs play critical roles in promoting the apoptosis [37]. Several Bcl family proteins, including the pro- and antiapoptotic groups, are already under the control of the JNK and/or p38 MAPK cascade [38]. Our study confirmed MAPK-signaling pathway activation after DEX intervention and induced changes in Bcl family proteins, with MAPK-signaling pathway activation leading to increased expression of the proapoptotic protein Bax and decreased expression of the antiapoptotic protein Bcl 2. Moreover, MAPK-signaling pathway activation is closely associated with ROS levels. Studies showed that the MAPK-signaling pathway is downstream of ROS, and ROS accumulation activates the MAPK-signaling pathway to induce apoptosis [39, 40]. NAC abolished the upregulation of p-ERK and p-JNK, key proteins in the MAPK pathway, thereby inhibiting the MAPK-signaling pathway [23]. Additional studies have shown that MAPKs can be activated by the curcumin to induce the production of endogenous ROS, thereby promoting apoptosis in cancer cells [41]. The MAPK/ERK-signaling pathway is also thought to be involved in osteoporosis [42]. Our study confirmed that DEX-induced osteoblast apoptosis may result from the increased ROS production induced via MAPK-signaling pathway activation.

To date, no single treatment has completely cured the necrosis of the femoral head. Previous studies have reported a method for autologous implanted MSCs to delay or avoid the collapse of the femoral head [43]. Coculture of MSCs with osteoblasts promotes osteoblast proliferation and osteogenic [44]. All the above studies confirmed the therapeutic effect of MSCs on necrosis of the femoral head. Moreover, MSC-derived exosomes can also reduce the risk of ONFH [14]. Liao et al. [45] found that BMSC-derived exosomes can multiply promote osteoblasts in the necrosis of the femoral head. Unlike direct stem cell transplantation, exosomes have the advantages of intrinsic homing effects, low immunogenicity, and effective molecular signaling stimulation [46]. However, exosomes, due to their low yield and complex extraction, cannot be applied on a large scale. Our previous studies have confirmed that the EMVs produced by continuous extrusion of cells has a similar structure and composition to exosomes, but its yield is tens of times the exosomes [16]. MSC-EMVs have been used widely in the various diseases. MSC-EMVs have been used as a drug delivery vehicle for a variety of cancers, including osteosarcoma, neuroblastoma, and breast cancer [47–49]. Furthermore, MSC-EMVs showed promising results in tissue repair. For example, the combination of hucMSC-EMVs with an injectable chitosan hydrogel injected into a mouse unhealthy cranial defect showed robust bone regeneration [50]. EMVs from MSCs (IONP-MSCs) combined with iron oxide nanoparticles (IONPs) reduces cardiomyocyte apoptosis and fibrosis to promote cardiac repair [18]. MSC-EMVs can facilitate repair in SCI [51]. However, the effect of MSC-EMVs on the ONFH has not been deeply studied. We demonstrated that hucMSC-EMVs reduced DEX-induced osteogenic cell apoptosis as well as ROS accumulation. Furthermore, MSC-EMVs inhibited DEX-induced activation of the
Figure 5: Continued.
FIGURE 5: Changes in apoptosis levels in MC3T3-E1 cells after hucMSC-EMVs and NAC intervention. (a) CCK-8 test for the viability of MC3T3-E1 cells after intervention with different concentrations of hucMSC-EMVs. (b and c) Apoptosis level of MC3T3-E1 cells after hucMSC-EMVs and NAC intervention. (d) Bax and Bcl 2 expression after intervention of hucMSC-EMVs. (e) Bax and Bcl 2 expression after NAC intervention by western blot. *p<0.05, **p<0.01, and ****p<0.0001 compared with control group. ^p<0.05, ^p<0.01, and ^p<0.0001 compared to the DEX group.
FIGURE 6: Oxidative stress levels in MC3T3-E1 cells after hucMSC-EMVs and NAC intervention. (a) ROS generation level of MC3T3-E1 cells after hucMSC-EMVs and NAC intervention (scale bar: 100 μm). (b) HO-1 and Nrf 2 expression levels in MC3T3-E1 cells after hucMSC-EMVs and NAC intervention by western blot. ∗p<0.05, ∗∗p<0.01, ∗∗∗p<0.001, and ∗∗∗∗p<0.0001 compared with control group. #p<0.05 and ##p<0.01 compared to the DEX group.
MAPK-signaling pathway and had the same effect as the ROS inhibitor NAC.

5. Conclusions

In conclusion, our study confirmed the critical role of MAPK-signaling activation as well as ROS accumulation in DEX-induced osteoblast apoptosis. In addition, we confirmed that hucMSC-EMVs can downregulate the MAPK signaling as well as ROS levels to attenuate the DEX-induced osteoblast apoptosis. We believe that MSC-EMVs may serve as a potential treatment measure for hormonal necrosis of the femoral head. Furthermore, further in vivo studies are needed to validate the results of this study.

Abbreviations

MSCs: Mesenchymal stem cell
DEX: Dexamethasone
hucMSC: Human umbilical cord MSC
EMVs: Exosome mimetic vesicles
ROS: Reactive oxygen species
NAC: N-acetyl-l-cysteine
NONFH: Nontraumatic osteonecrosis of the femoral head
THAs: Total hip replacement
FBS: Fetal bovine serum
CCK-8: Cell Counting Kit-8.

Data Availability
The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical Approval
This study did not involve clinical samples and animal experiments, and all did not require ethical approval.

Conflicts of Interest
The authors declare that they have no conflicts of interest.

Authors’ Contributions
ZXZ and HXL designed the study; ZXZ, TM, JKW, YML, and JYL collected and analyzed the data; ZXZ, ZYW, and WQC drafted the initial manuscript; PG, XW, LMJ, and DWH revised the article critically; HXL, JHL, and DWH reviewed and edited the article. All authors approved the final manuscript.

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Supplementary Materials
Table S1: antibodies used for different experiments in this report. (Supplementary Materials)

References


