Research Article
Silica/OCP Affects the Viability of Osteoblasts through ROS-Induced Autophagy

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Objective. To explore the effects of silicone gel nanoparticles modified with octacalcium phosphate on the proliferation of osteoblasts and autophagy.

Method. Silica/OCP was prepared in vitro, and the quality of the sample preparation was tested through characterization experiments. The osteoblast cell line (hFOB1.19) was treated with silica/OCP, autophagy inhibitor (3-methyladenine (3-MA)), and silica/OCP+3-MA, respectively. The proliferation of hFOB1.19 cells was detected through the methylthiazolyldiphenyl-tetrazolium bromide (MTT) kit. Flow cytometry was used to detect the cell apoptosis. The change in protein beclin1 and P62 expression in hFOB1.19 cells was observed in Western blot. An ROS detection kit was used to detect the content of reactive oxygen species in hFOB1.19 cells.

Results. Silica/OCP was a sphere with a particle size of 50 nm to 130 nm and had an OCP phase in electron projection microscopy and X-ray diffraction techniques. The results indicated that OCP successfully modified silica and the material was successfully prepared. An MTT kit and flow cytometry test showed that the cell viability of the cells treated with silica/OCP increased significantly (P < 0.05), and the intracellular apoptosis phenomenon was significantly decreased (P < 0.05) compared to the control group. Moreover, the inhibition of cell viability and promotion of apoptosis caused by the autophagy inhibitor 3-MA can be rescued. Western blotting demonstrated that the protein level of beclin1 in osteoblasts reached the highest after six hours of treatment with silica/OCP, and the protein level of p62, the substrate protein of autophagy, reached the lowest. At the same time, treatment of cells with the autophagy inhibitor 3-MA and silica/OCP+3-MA found that the protein levels of beclin1 and p62 in the silica/OCP+3-MA group were adjusted back compared to the 3-MA group. After adding the autophagy inhibitor, the reactive oxygen content in the cell was significantly increased (P < 0.05) in the silica/OCP group. In the presence of intracellular reactive oxygen inhibitors catalase and silica/OCP, the cell viability of osteoblasts was significantly lower than that of the silica/OCP group but significantly higher than that of the silica/OCP+3-MA group. The apoptosis level of the silica/OCP+catalase group was also significantly lower than that of the silica/OCP+3-MA group (P < 0.05) but was significantly higher than that of the silica/OCP group (P < 0.05). Conclusion. Silica/OCP nanoparticles can upregulate the level of autophagy in osteoblasts and promote the proliferation of osteoblasts.

1. Introduction

Silica gel nanoparticles (silica) are the most potential new nanomaterials in modern biotechnology and biomedicine. Silica gel nanoparticles can effectively inhibit connective tissue getting into defective bone, thus mediating bone regeneration and promoting the potential of bone tissue regeneration [1]. Feng et al. showed that the surface of silica nanoparticles modified with octacalcium phosphate (OCP) significantly promoted the activity and proliferation of MG-63 osteoblasts and the expression of alkaline phosphatase and osteocalcin protein [2]. However, there is no report on the application of silica/OCP in the field of osteoporosis. Autophagy plays a vital role in cell energy balance, quality control, structural reconstruction, and immunity [3, 4]. According to the way substrates enter lysosomes, autophagy can be divided into three types: macroautophagy, microautophagy, and molecular chaperone-mediated autophagy.
Macroautophagy is the most frequently occurring autophagy and is the most profoundly studied autophagy form at present. Therefore, autophagy is generally referred to macroautophagy. When cells are stimulated by hypoxia, oxidative stress, and endoplasmic reticulum damage, it can induce autophagy and then respond to external stimulation to prevent apoptosis [5–7]. Previous studies have shown that autophagy makes cells survive under various pressures and plays an important protective role in maintaining the viability of osteoblasts [8, 9]. Among them, microtubule-associated protein light chain 3 (LC3), beclin1, and p62 are important biological signals to recognize autophagy [10]. In the process of autophagy, autophagosomes phagocytize proteins and organelles in the cytoplasm. The precursor LC3 is synthesized and processed to form LC3I, which is conjugated with phosphatidylethanolamine fixed on the autophagosome membrane to form LC3II. When autophagy is formed, LC3II will decrease and LC3II will increase. Therefore, the activity of autophagy can be characterized by the activity of LC3II [11]. In addition, p62 bound to the substrate can be degraded by proteolytic enzymes during lysosomal degradation. Therefore, the increase in p62 level is usually considered a sign of inhibition of autophagy. Beclin1 is also an essential protein for the formation of autophagosomes. Under the joint action of the ULK1 complex, beclin1 forms a nuclear isolation membrane (phagophore) somewhere in the cell. Subsequently, the ATG16L1 complex was recruited to the isolation membrane to mediate LC3 lipoylation, thus making the isolation membrane extend continuously and encapsulating the substances that need to be degraded in the cell. Finally, the membrane closed to form the autophagosome [12].

Mitochondria produce reactive oxygen species (ROS) during cell metabolism. Under normal conditions, reactive oxygen species in cells participate in regulating energy metabolism in the body. When cells are subjected to stress, the content of reactive oxygen species in cells will increase significantly, induce apoptosis and death, and then cause diseases in the body [13]. Related studies have shown that increased oxidative stress in older people and mice can cause diseases in the body [13]. Related studies have shown that increased oxidative stress in older people and mice can cause diseases in the body [13].

2. Materials and Methods

2.1. Main Reagents and Instruments. Dulbecco’s Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from the HyClone company of the United States, and cyanocycline was purchased from Dingguo, Beijing. Inverted fluorescence microscopy (IX71) was from Olympus, Japan. An ROS detection kit and MTT cell proliferation and toxicity detection kit were bought from Biyuntian, Shanghai. A BCA protein assay kit was purchased from Wuhan Bost Biotechnology Co., Ltd. (China). ModFit LT 30 flow cytometry was from BD Biosciences, Franklin. P62, the antibody against beclin1, and horseradish peroxidase-labeled goat anti-rabbit antibodies were purchased from American Cell Signaling Technology (Danvers, USA). Rapamycin and 3-methyladenine were purchased from Selleck (Houston, TX, USA), and catalase was purchased from Shanghai Biyuntian company. Western and IP cracking solution was from Biyuntian, Shanghai; a protease inhibitor was from Thermo Fisher Scientific, USA.

2.2. Cell Culture and Treatment. Human osteoblast line hFOB1.19 cells were purchased from the cell bank of Shanghai Chinese Academy of Sciences. The cells were cultured in a high-glucose DMEM supplemented with 10% FBS and placed in a constant incubator with 5% CO2 and at 33.5°C. The cells were treated with silica/OCP for a different time after logarithmic growth. Rapamycin (3 μM), 3-methyladenine (5 mM), and catalase (500 U/ml) were added into the cell culture at time points.

2.3. Preparation and Characterization of Silica/OCP Nanoparticles. Tetraethyl silicate, ammonia water, and deionized water were added into 180 ml dehydroethanol at a molar ratio of 1:2:5. The silica nanoparticle suspension was prepared by stirring at 200 rpm for 6h at 35°C. The pH value of silica nanoparticle suspension was adjusted to 6.5, and calcium nitrate and calcium phosphate solution was added. The suspension was continued being stirred at 200 rpm for 3h at 40°C and then dried to obtain silica/OCP nanoparticles.

10 mg of dried silica/OCP nanoparticles was dissolved in distilled water and evenly smeared on the copper plate rich in nitrocellulose. An electron transmission microscope was utilized to observe the morphology of silica/OCP nanoparticles. The size distribution of the prepared silica/OCP nanoparticles was measured by the dynamic light scattering method at 25°C, and the composition of silica/OCP nanoparticles was demonstrated by X-ray diffraction.

2.4. Cell Viability Test. hFOB1.19 cells were seeded into 96-well plates with a density of 5 * 104. After 36 hours of incubation in an incubator, add an autophagy inhibitor (5 mM) and intracellular reactive oxygen species inhibitor (500 U/ml) to each well for 1 hour, and then, incubate cells with silica/OCP for 6 hours. The supernatant was removed, and then, 90 μl of fresh culture medium was added into each well. 10 μl of methylthiazolyldiphenyl-tetrazolium bromide (MTT) solution was added for 4h. The supernatant was then discarded, and 100 μl of dimethyl sulfoxide was added to each well for 15 min at room temperature. Then, the absorbance of each well was measured at 490 nm by ELISA.

2.5. Analysis of Apoptosis. After being treated with the autophagy inhibitor (5 mM) and intracellular reactive oxygen species inhibitor (500 U/ml) for 1h, then incubate cells with silica/OCP for 6h. The cells were collected and washed twice with precooled PBS. 100 μl binding buffer, 5 μl Annexin V-FITC, and 10 μl PI staining solution were added.
Then, the cells could react for 10-15 min in the dark at room temperature. The apoptosis (BD Biosciences, Franklin) was analyzed by ModFit LT 3.0 flow cytometry. The following criteria were used to distinguish different subpopulations: Q3, live cells (FITC-/PI-); Q4, early apoptotic cells (FITC+/PI-); Q2, (FITC+/PI+); and Q1, necrotic cells (FITC-/PI+).

2.6. Determination of Reactive Oxygen Species in Cells. A ROS detection kit monitored the ROS level of osteoblasts. The cells in the 6-well plate were incubated with rapamycin or 3-methyladenine for 1 hour before being treated with silica/OCP. The cells in the 6-well plate were incubated with silica/OCP for 6 hours. The cells were incubated with a fluorescent probe for reactive oxygen species at 37°C for 20 minutes and then washed with PBS. The level of ROS was measured by flow cytometry.

2.7. Western Blotting. The treated hFOB1.19 cells were collected and lysed in the lysis buffer containing a protease inhibitor. The extract was centrifuged at 13000 rpm at 4°C; then, the supernatant was collected. The protein concentration was determined by the BCA protein assay. Proteins were separated by SDS-PAGE electrophoresis and transferred to the PVDF membrane (Millipore, USA). Then, the membrane was incubated with anti-P62 (1:1000), beclin1 (1:1000), and GAPDH (1:2000) at 4°C overnight. Next, the membrane was incubated with a goat anti-rabbit antibody (1:5000) coupled with horseradish peroxidase for 1 hour at room temperature. The protein signal was detected by the chemiluminescence system (DNR MF-ChemiBIS 3.2). The ImageJ image processing program was used to quantify the gray level of the strip.

2.8. Statistical Analysis. All experiments were repeated three times. Quantitative data are expressed as an average ± SD. All statistical analyses were performed by using SPSS 24.0 software (IBM, USA) using ANOVA. The t-test was used to determine statistical significance (P < 0.05).

3. Results

3.1. Characterization of Silica/OCP Nanoparticles. The prepared silica/OCP nanoparticles are homogeneous spheres (Figure 1(a)) under the electron transmission microscope, Figure 1: Characterization of silica/OCP nanoparticles: (a) silica/OCP nanoparticles were observed by TEM; (b) particle size distribution of silica/OCP nanoparticles; (c) the silica/OCP nanoparticle composition was observed by X-ray diffraction.
and the particle size ranges from 50 nm to 130 nm (Figure 1(b)). X-ray diffraction results showed that the OCP phase was detected in silica/OCP nanoparticle suspension (Figure 1(c)), indicating that OCP modified the surface of silica nanoparticles.

3.2. Silica/OCP Upregulated the Level of Autophagy and Cell Viability in Osteoblasts. To explore whether silica/OCP can upregulate the autophagy level of osteoblasts in hFOB1.19 cells, we pretreated the cells with the autophagy inhibitor 3-MA to decrease autophagy level. The results showed that after treating the cells with silica/OCP alone, the cell viability was upregulated \( (P < 0.05) \), while the cell viability of the silica/OCP+3-MA group was lower than that of the silica/OCP group but higher than that of the 3-MA group \( (P < 0.05) \) (Figure 2(a)). At the same time, flow cytometry showed that compared with the normal group, the silica/OCP group induced fewer apoptotic cells and could rescue the apoptosis caused by 3-MA (Figures 2(b) and 2(c)). Therefore, we demonstrated that silica/OCP could upregulate the autophagy level and promote osteoblast viability to some extent.

3.3. Silica/OCP Upregulated Autophagy Level in hFOB1.19 Cells in a Time-Dependent Manner. To determine whether silica/OCP can induce autophagy, we applied western blotting to detect the protein level of p62, an autophagy
substrate, and beclin1, an important component of the autophagy membrane. The results indicated that the protein levels of p62 and beclin1 changed significantly with the prolongation of incubation time (0, 2, 4, 6, and 8 h) in hFOB1.19 cells. The protein level of beclin1 gradually increased from 3 hours and reached a peak at 6 hours (Figures 3(a) and 3(b)). The protein content of p62, the autophagy substrate, had an opposite trend to that of beclin1. The protein level of p62 gradually decreased from 3 hours and reached the lowest point at 6 hours (Figures 3(a) and 3(b)). These results indicated that silica/OCP-induced autophagy reached the maximum level in hFOB1.19 cells at about 6 hours. It may affect the occurrence of autophagy by upregulating the autophagosome protein beclin1. However, 3-MA significantly inhibited silica/OCP-induced autophagy (Figures 3(c) and 3(d)).

3.4. The Autophagy of hFOB1.19 Cells Is Caused by the Increase in ROS Induced by Silica/OCP.

We detected the change of ROS content in the presence of an autophagy activator and autophagy inhibitor to examine the correlation...
among silica/OCP, ROS, and autophagy. Osteoblasts were incubated with rapamycin or 3-methyladenine for 1 hour before being treated with silica/OCP. The fluorescence intensity of DCF is detected by flow cytometry to show the level of ROS in hFOB1.19 cells. The results showed that the ROS level was significantly decreased \( (P < 0.05) \). On the contrary, when autophagy was inhibited with 3-MA, the level of ROS increased significantly \( (P < 0.05) \) (Figures 4(c) and 4(d)). These results suggest that autophagy can protect osteoblasts by reducing the level of ROS.

3.5. ROS Inhibitors Inhibit the Increase in Cell Activity Induced by Silica/OCP in hFOB1.19 Cells. Based on above experiments, we believe that silica/OCP can inhibit apoptosis by upregulating the content of intracellular free oxygen. It can increase the level of autophagy and the activity of hFOB1.19 cells through intracellular oxidative stress. To verify the critical role of reactive oxygen species in the viability of osteoblasts induced by silica/OCP, we first studied the viability of osteoblasts by the MTT assay. In the presence of catalase and silica/OCP, the survival rate of hFOB1.19 cells was significantly lower than that of the control group \( (P < 0.05) \), which was consistent with that of the 3-MA group (Figure 5(a)). The same results were obtained by flow cytometry analysis. The number of apoptotic cells treated with catalase and silica/OCP was significantly higher than that treated with silica/OCP alone \( (P < 0.05) \) (Figures 5(b) and 5(c)). In conclusion, silica/OCP can increase the activity of osteoblasts by upregulating the content of free oxygen in osteoblasts.

4. Discussion

As we all know, macroautophagy exists in all cells, which is considered to regulate the quality of intracellular macromolecules and play a crucial role in maintaining the body’s homeostasis [4]. Autophagy is usually upregulated under some stress [15]. However, if it is overregulated, the
autophagosome will phagocytize the normal macromolecules in the cell, eventually leading to cell death. Autophagy is also considered a type II form of programmed cell death in the body [16]. However, under some mild stimulation conditions, the body can promote cell survival and avoid apoptosis by slowing down some stress-inducing factors, moderately inducing autophagy, and eliminating unfavorable factors [17]. At the same time, the metabolites decomposed during autophagy can be transferred to cells as nutrients to meet their energy needs under pressure [18, 19]. This process is important for some terminally differentiated cells (such as osteoblasts and osteoclasts) to survive [20]. Many recent studies have shown that autophagy can promote the survival ability of osteoblasts under various pressures [21]. In addition, it has been fully demonstrated that GC-induced autophagy is involved in the regulation of cell viability.

Free oxygen in cells is produced by the standard metabolism of the body [22]. However, with the in-depth study of the pathogenic mechanism of GCs, it is found that GCs can cause excessive accumulation of free oxygen in cells by blocking the function of mitochondria, leading to the occurrence of oxidative stress [23]. Similarly, our results showed that the level of ROS increased in hFOB1.19 cells treated

![Graph](image-url)
with silica/OCP. Excessive accumulation of ROS may interfere with cell homeostasis, leading to cell damage and death [24]. In this study, we found that when the content of free oxygen in cells increased sharply, the body would have an oxidative stress reaction, which inhibited the differentiation of osteoblasts and their proliferation activity to a certain extent and reduced bone formation. These results are consistent with previous reports [25, 26]. At the same time, when we treated cells with silica/OCP, we observed that the levels of autophagy and cell viability were also significantly improved by utilizing the MTT kit and flow cytometry.

However, studies have shown that normal levels of ROS in cells act as intracellular signaling molecules, and appropriate ROS can regulate cell viability by upregulating autophagy [21, 27]. According to previous reports, we hypothesized that silica/OCP increases osteoblast activity through intracellular ROS-induced autophagy. We treated osteoblasts with silica/OCP in the presence or absence of the ROS scavenger catalase to confirm this conjecture. The results showed that catalase significantly inhibited the increase in cell activity and autophagy induced by silica/OCP. In addition, it has been reported that the accumulation of autophagy activity can substantially reduce the content of intracellular free oxygen [28]. In our study, upregulation of Rap autophagy decreased intracellular ROS in osteoblasts treated with silica/OCP, while 3-MA pretreatment produced the opposite result. All in all, our data show that silica/OCP can increase the level of autophagy by upregulating the content of ROS in osteoblasts. The initiation of autophagy can eliminate the oxidative stress response of the body and then reduce the level of apoptosis, thus protecting the proliferation of osteoblasts.

Data Availability
The data and materials used to support the findings of this study are included within the published article.

Conflicts of Interest
The authors declare that there are no conflicts of interest regarding the publication of this paper.

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