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A Comparison of the Biological Activities of Human Osteoblast hFOB1.19 Between Iron Excess and Iron Deficiency

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Abstract Bone metabolism has a close relationship with iron homeostasis. To examine the effects of iron excess and iron deficiency on the biological activities of osteoblast in vitro, human osteoblast cells (hFOB1.19) were incubated in a medium supplemented with 0–200 μmol/L ferric ammonium citrate and 0–20 μmol/L deferoxamine. The intracellular iron was measured by a confocal laser scanning microscope. Proliferation of osteoblasts was evaluated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay. Apoptotic cells were detected using annexin intervention V/PI staining with a flow cytometry. Alkaline phosphatase (ALP) activity was measured using an ALP assay kit. The number of calcified nodules and mineral area was evaluated by von Kossa staining assay. The expressions of type I collagen and osteocalcin of cultured osteoblasts were detected by reverse transcriptase polymerase chain reaction and Western blot. Intracellular reactive oxygen species (ROS) was measured using the oxidation-sensitive dye 2,7-dichlorofluorescin diacetate by flow cytometry. The results indicated that excessive iron inhibited osteoblast activity in a concentration-dependent manner. Low iron concentrations, in contrast, produced a biphasic manner on osteoblasts: mild low iron promoted osteoblast activity, but serious low iron inhibited osteoblast activity. Osteogenesis was optimal in certain iron concentrations. The mechanism underlying biological activity invoked by excessive iron may be attributed to increased intracellular ROS levels.

Keywords Iron ion · Osteoblast · Deferoxamine · Proliferation · Mineralization

Abbreviations

FAC	Ferric ammonium citrate
DFO	Deferoxamine
ALP	Alkaline phosphatase
COL-I	Type I collagen
OC	Osteocalcin
RT-PCR	Reverse transcriptase polymerase chain reaction
ROS	Reactive oxygen species

Introduction

Iron is an important component of hemoglobin, myoglobin, cytochrome, and some atmungsferments and participates in the transfer and exchange of oxygen and carbon dioxide and promotes the transportation of lipids in the blood [1, 2]. Excess iron, however, has more significant toxic effects. Iron toxicity is presented by catalyzing free radical formation [3, 4]; iron is also an essential nutrient for pathogenic microorganism and neoplastic cell [5].

In recent years, preclinical and clinical studies have demonstrated a close relationship in iron metabolism and bone metabolism. In 2006, Weinberg [6] pointed out that iron loading was a risk factor for osteoporosis. In 2008, Weinberg [7] again reported the role of iron in osteoporosis. Clinical studies demonstrated that there was a higher incidence of osteoporosis in patients with iron overload such as hereditary hemochromatosis, thalassemia, and sickle cell

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anemia [8–12]. In addition, iron overload through diet or injection of colloidal iron can result in osteoporosis in rats [13–15]. Iron deficiency also can cause abnormal bone metabolism. Iron deficiency in postmenopausal women appears to be associated with decreased bone density [16, 17]. In animal studies, moderate to severe dietary iron deficiency results in altered bone morphology/micro-architecture and decreased density [18, 19]. Thus, iron deficiency as well as iron overload has harmful effects on bone metabolism.

Osteoblasts are the main functioning cells in bone metabolism and participate in bone repair and reconstruction. They also produce calcium nodules and collagen, both critical components in bone mechanical support. In vitro experiments demonstrated that excess iron could inhibit the biological activity of osteoblasts [20–22]. Low iron, in contrast, inhibits osteoblastogenesis in vitro [23]. However, the effects of excessive iron and low iron on osteoblasts are not fully clear under the same experimental condition. The objective of our study is to examine the effects of iron of different concentrations on osteoblasts under the same experimental condition.

Material and Methods

Material

The human fetal osteoblast cell line (hFOB1.19) was obtained from the Institute of Biochemistry and Cell Biology of Shanghai (Shanghai, China). Ferric ammonium citrate (FAC) was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Deferoxamine (DFO) was purchased from Novartis Pharma (Basel, Switzerland). Dulbecco's modified Eagle's medium-F_{12} (DMEM-F_{12}) and fetal bovine serum were from Gibco (Grand Island, NY, USA). Phen Green FL was from Molecular Probes (Eugene, USA). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and 2,7-dichlorofluorescin diacetate (DCF-DA) were from Sigma (St. Louis, MO, USA). Alkaline phosphatase assay kit was from Jiancheng (Nanjing, China). TRZOL reagent and apoptosis assay kit were from Invitrogen (Carlsbad, CA, USA). von Kossa staining kit was from GenMed (Boston, USA). Primary antibody for type I (COL-I) collagen was from Abcom, USA. Primary antibody for osteocalcin (OC) was from Santa Cruz, USA.

Cell Cultures and Treatments

The hFOB1.19 cells were maintained in DMEM-F_{12} supplemented with 10 % fetal bovine serum and 3 % G418 disulfate solution in a humidified atmosphere of 5 % CO_2 in air at 34 °C. The medium was replenished every 2–3 days. After reaching 70–80 % confluence, cells were passaged by treatment with 0.05 % trypsin. FAC and DFO were added to the media at final concentrations of 50, 100, or 200 μmol/L FAC as treatment of excess iron group and 5, 10, or 20 μmol/L DFO as treatment of low iron group, respectively. Deionized water was added to the media as control group (0 μmol/L).

Measurement of Intracellular Iron

The cells were seeded on coverslips for fluorescence analysis of iron ion. Briefly, after treatment of FAC and DFO for 48 h, the cells were washed twice with phosphate-buffered saline (PBS) and incubated with Phen Green FL away from light at 34 °C in a humidified atmosphere containing 5 % CO_2 for 30 min. Next, the cells were washed twice with PBS to remove the unbound fluorescent indicator and then incubated with the culture medium for another 15 min. A confocal laser scanning microscope (Leica, Germany) was used to measure the green fluorescence of Phen Green FL when excited at 488 nm and emitted at 521 nm.

Cell Proliferation Assay

Cell proliferation was examined by MTT assay. Briefly, the hFOB1.19 cells were treated with FAC and DFO for 48 h; thereafter, MTT dye solution was added. After 4 h incubation, the supernatant was removed and DMSO was added to solubilize the MTT. The optical density (OD) was measured at a wavelength of 570 nm using microplate reader (BioTek, USA).

Cell Apoptosis Assay

The hFOB1.19 cells were treated with FAC and DFO for 48 h. Annexin-U-FIFC and PI were added to cell suspension and the cells were incubated for 15 min. Fluorescence intensity was measured using flow cytometer (Becton Dickinson, San Jose, CA) in 1 h.

ALP Activity Assay

The hFOB1.19 cells were treated with FAC and DFO for 10 days; thereafter, decomposition fluid was added. Aliquots of supernatant were subjected to alkaline phosphatase (ALP) activity and protein measurement using an ALP kit and BCA protein assay kit, respectively. All results were normalized by protein content. The OD was measured at a wavelength of 520 nm using a microplate reader.

von Kossa Staining Assay

The hFOB1.19 cells were treated with FAC and DFO for 3 days; thereafter, the culture media were replaced with a medium containing von Kossa calcification fluid every other day. At day17, cells were stained by von Kossa calcification. Calcification area was calculated as percentage to the entire area using a HPIAS 2100 high-resolution pathology report analysis system.

RT-PCR

Cells were treated with FAC and DFO for 72 h prior to total RNA extraction using a TRZOL reagent. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed with a reverse transcription kit from Promega (Madison, WI, USA) and Taq polymerase (Madison, WI, USA). The primer sequences were: β-actin: 5′-TCCTGTGGCATCCACGAAACT-3′ (forward) and 5′-GAAGCATTTGCGGTGGACGAT-3′ (reverse); COL-I: 5′-GATGCCAATGTGGTTCGTG-3′ (forward) and 5′-TTCTTGCGGCTGCCCTCT-3′ (reverse); and OC: 5′-GGTGCAGAGTCCAGCAAAGGTG-3′ (forward) and 5′-AGCTCACACACCTCCCTCCTG-3′ (reverse). The RT-PCR cycling conditions were 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s at 30 cycles. Eight microliters of each PCR product was loaded to a 1.5 % agarose gel with ethidium bromide for electrophoresis and visualization under UV light. The images were analyzed with Image J software.

Western Blot

After FAC and DFO were added to wells, respectively, for 72 h, total protein was extracted using a RIPA buffer and separated on 6 % SDS gel prior to transfer onto polyvinylidene difluoride membranes. The membranes were blocked in 5 % (*m*/*v*) milk dissolved in Tris-buffered saline with 0.05 % (*w*/*v*) Tween-20 (TBS-T) and incubated overnight at 4 °C with a COL-I primary antibody (1:5,000) or an OC primary antibody (1:1,000). After washing three times with TBS-T at room temperature, the membranes were incubated for 1 h with an HRP-conjugated secondary antibody (1:500) and visualized using an enhanced chemiluminescence system (Amersham Bioscience, Piscataway, NJ, USA). The images were analyzed with Image J software.

Flow Cytometric Determination of ROS

The hFOB1.19 cells were incubated in the dark for 30 min in Krebs-Ringer solution containing 10 μmol/L of 2,7-DCF-DA, and they were treated as indicated for 2 h. The cells were washed three times with PBS and immediately analyzed by flow cytometry (Cytomics FC500, Beckman

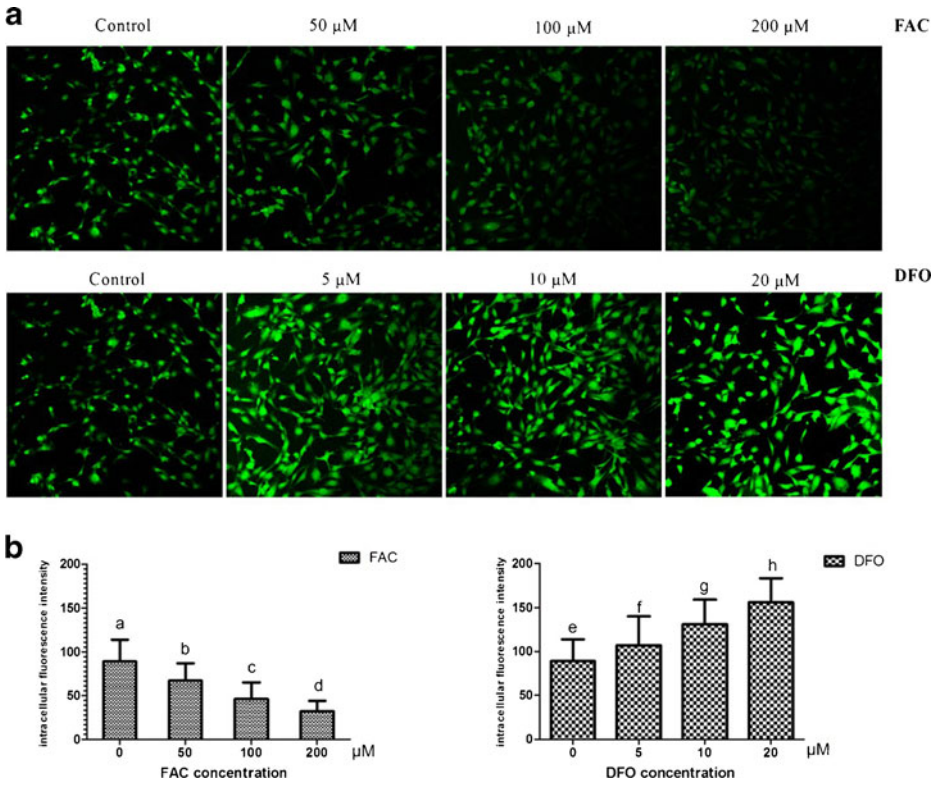

Fig. 1 Confocal microscopy analysis of iron concentration in osteoblast. **a** The iron fluorescence was measured with a confocal laser scanning microscope. Representative microscopic fields are shown (×20). **b** The fluorescence intensity which correlated with intracellular iron concentration was significantly weakened with increasing of FAC concentration and enhanced with increasing of DFO concentration. The means±SD of three independent experiments are shown. *Means with different letters* were significantly different ($p<0.05$)

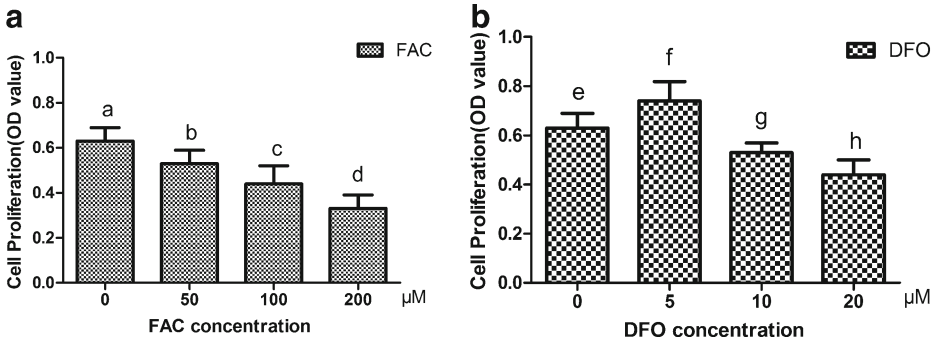

Fig. 2 The proliferation of osteoblast after exposure to FAC and DFO at various concentrations. **a** The proliferation was inhibited with the increasing of FAC in a concentration-dependent manner. **b** The proliferation was promoted at 5 μmol/L of DFO, but inhibited at 10 and 20 μmol/L. The results were expressed as the means±SD. *N*=5 wells per treatment per independent study. *Means with different letters* were significantly different ($p<0.05$)

Coulter, USA) with 488-nm excitation. The signals were obtained using a 525-nm band-pass filter for DCF. Each determination is based on the mean fluorescence intensity of 10,000 cells.

Statistical Analysis

Data are expressed as mean±SD and analyzed with a one-way analysis of variance with post hoc analysis using SPSS version 15.01 for Windows. A p value less than 0.05 was considered significant.

Result

Effects of FAC and DFO on Intracellular Iron

When these hFOB1.19 cells were exposed to various concentrations of FAC and DFO for 48 h, the fluorescence intensity which correlated with intracellular iron concentration was significantly weakened with increasing of FAC concentration and enhanced with increasing of DFO concentration ($p<0.05$ for all comparisons; Fig. 1). This indicated that FAC could effectually increase intracellular iron, and DFO could effectually decrease intracellular iron.

Effects of Iron on the Proliferation of Osteoblasts

FAC decreased the proliferation of the hFOB1.19 cells in a concentration-dependent manner ($p<0.05$ for all comparisons; Fig. 2). In contrast, DFO exposure increased the proliferation of the hFOB1.19 cells at a concentration of 5 μmol/L, but decreased at higher concentrations or 10 and 20 μmol/L ($p<0.05$ for all comparisons; Fig. 2).

Effects of Iron on the Apoptosis of Osteoblasts

FAC increased apoptosis rate of the hFOB1.19 cells in a concentration-dependent manner ($p<0.05$ for all comparisons; Fig. 3). In contrast, DFO significantly decreased apoptosis rate at 5 and 10 μmol/L, but increased at a higher concentrations of 20 μmol/L ($p<0.05$ for all comparisons; Fig. 3).

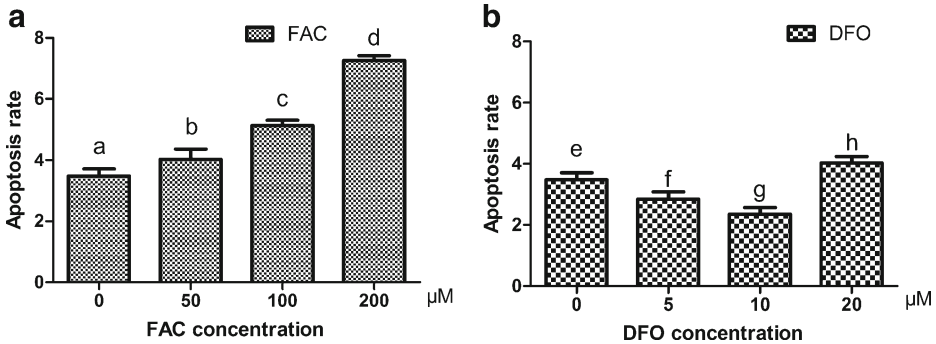

Fig. 3 The apoptosis rate of osteoblast after exposure to FAC and DFO at various concentrations. **a** The apoptosis rate was increased with the increasing of FAC in a concentration-dependent manner. **b** The apoptosis rate was decreased at 5 and 10 μmol/L of DFO, but increased at 20 μmol/L. The results were expressed as the means±SD. *N*=3 wells per treatment per independent study. *Means with different letters* were significantly different ($p<0.05$)

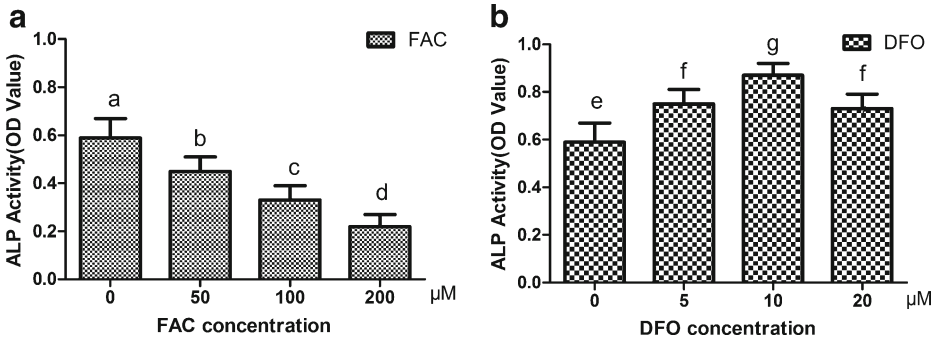

Fig. 4 ALP activity of osteoblast after exposure to FAC and DFO at various concentrations. **a** ALP activity was decreased with the increasing of FAC in a concentration-dependent manner. **b** ALP activity was increased at 5, 10, and 20 μmol/L groups of DFO compared to control, but was decreased in 20 μmol/L compared to 10 μmol/L. The results were expressed as the means±SD. N=3 wells per treatment per independent study. *Means with different letters* were significantly different (p<0.05)

Effects of Iron on Differentiation of Osteoblasts

ALP activity of the hFOB1.19 cells was decreased by FAC in a concentration-dependent manner (p<0.05 for all comparisons; Fig. 4). In contrast, DFO increased ALP activity at 5, 10, and 20 μmol/L (p<0.05 for various concentration groups compared to control), but ALP activity was decreased in 20 μmol/L compared to 10 μmol/L (p<0.05; Fig. 4).

Effects of Iron on Mineralization Function of Osteoblasts

von Kossa staining showed that FAC inhibited the mineralization function of the osteoblasts. Both the mineralized surface area and the number of mineralized nodules of osteoblasts were decreased with the increasing of FAC concentration (p<0.05 for all comparisons; Fig. 5). DFO increased the mineralized surface area and the number of mineralized nodules at 5 μmol/L (p<0.05 compared to control), has no significant effect at 10 μmol/L (p>0.05, compared to control), and decreased at 20 μmol/L (p<0.05 compared to control; Fig. 5).

Effects of Iron on COL-I and OC Expression

FAC decreased mRNA expression of COL-I and OC in a concentration-dependent manner (p<0.05 for all comparisons;

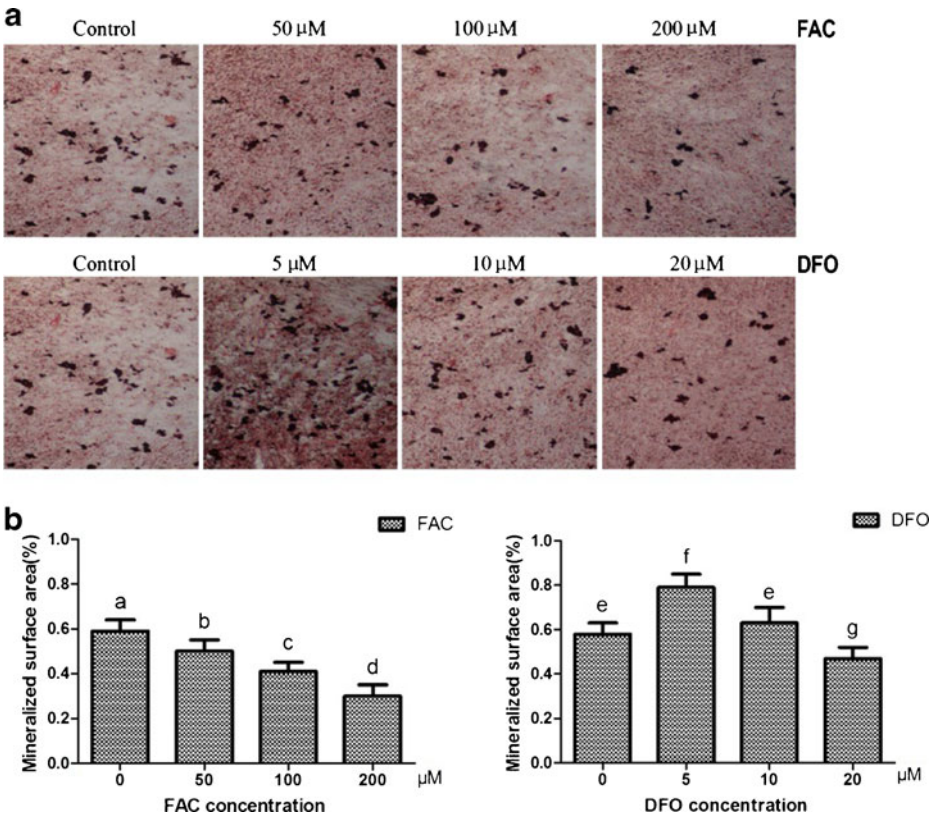

Fig. 5 Mineralization function of osteoblast after exposure to FAC and DFO at various concentrations. **a** Mineralized nodules by von Kossa staining (×100). **b** Average percentage of mineralized surface area of the osteoblasts. The number of mineralized nodules and the mineralized surface area decreased with the increasing of FAC in a concentration-dependent manner. The number of mineralized nodules and the mineralized surface area was increased at 5 μmol/L of DFO, not significant change at 10 μmol/L, and decreased at 20 μmol/L. The results were expressed as the means±SD. N=3 wells per treatment per independent study. *Means with different letters* were significantly different (p<0.05)

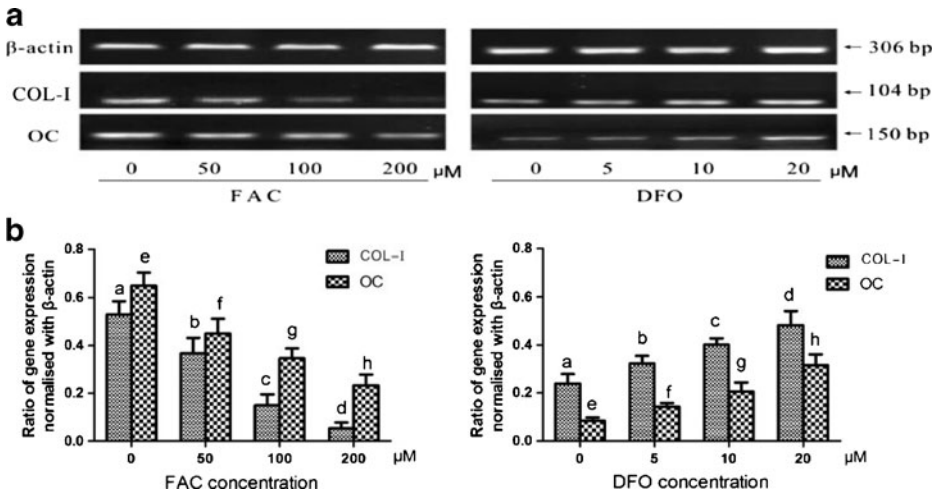

Fig. 6 The gene expression of COL-I and OC of osteoblast after exposure to FAC and DFO at various concentrations. **a** RT-PCR amplification of β-actin, COL-I, and OC. **b** The mRNA expression of COL-I and OC was decreased with the increasing of FAC in a concentration-dependent manner. The mRNA expression of COL-I and OC was increased with the increasing of DFO in a concentration-dependent manner. The results were expressed as the means±SD. N=3 wells per treatment per independent study. *Means with different letters* were significantly different (p<0.05)

Fig. 6). DFO increased the mRNA expression of COL-I and OC in a concentration-dependent manner (p<0.05 for all comparisons; Fig. 6). Western blot showed the same pattern of FAC and DFO effects on the expression of COL-I and OC at a protein level (Fig. 7).

Effects of Iron on the Production of ROS

Intracellular reactive oxygen species (ROS) in FAC groups was measured using the oxidation-sensitive dye DCF-DA by flow cytometry. Our data show that FAC increased intracellular ROS of the hFOB1.19 cells in a concentration-dependent manner (p<0.05 for all comparisons; Fig. 8).

Discussion

In present study, we examined proliferation, apoptosis, differentiation, and mineralization of osteoblast hFOB1.19 treated with FAC or DFO at different concentrations. The iron content of the treated cells was measured by a confocal microcopy measurement. Our results confirmed iron excess or iron deficiency in osteoblast by FAC or DFO treatment. The current study demonstrated that excessive iron inhibited osteoblast activity. Low iron concentrations, in contrast, produced a biphasic action on osteoblasts: mild low iron was beneficial to survival and function of osteoblast, but severely low iron produced adverse effect. The mechanism underlying biological activities invoked by excessive iron may attribute to increased intracellular ROS levels.

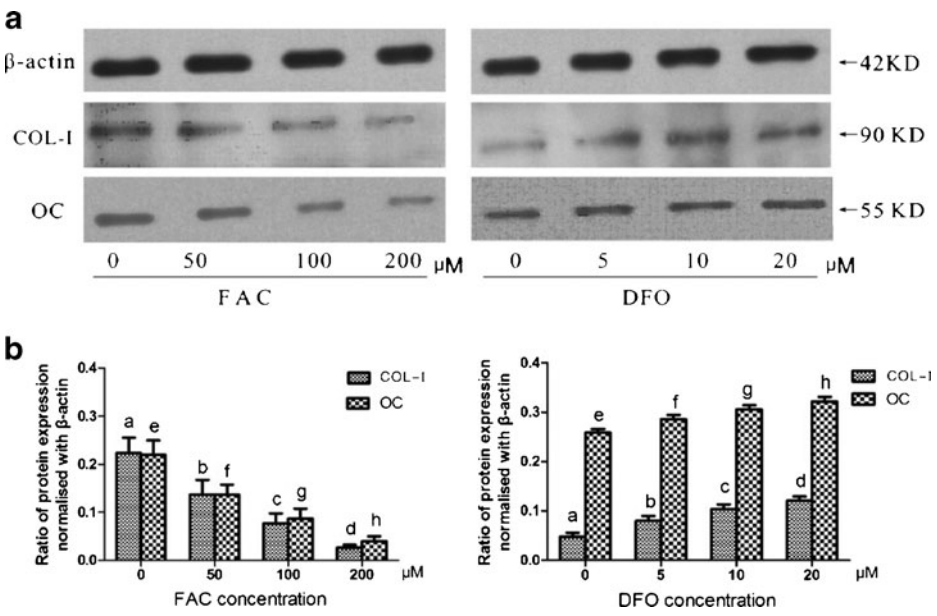

Fig. 7 The protein expression of COL-I and OC of osteoblast after exposure to FAC and DFO at various concentrations. **a** Western blots of β-actin, COL-I, and OC. **b** The protein expression of COL-I and OC was decreased with the increasing of FAC in a concentration-dependent manner. The protein expression of COL-I and OC was increased with the increasing of DFO in a concentration-dependent manner. The results were expressed as the means± SD. N=3 wells per treatment per independent study. *Means with different letters* were significantly different (p<0.05)

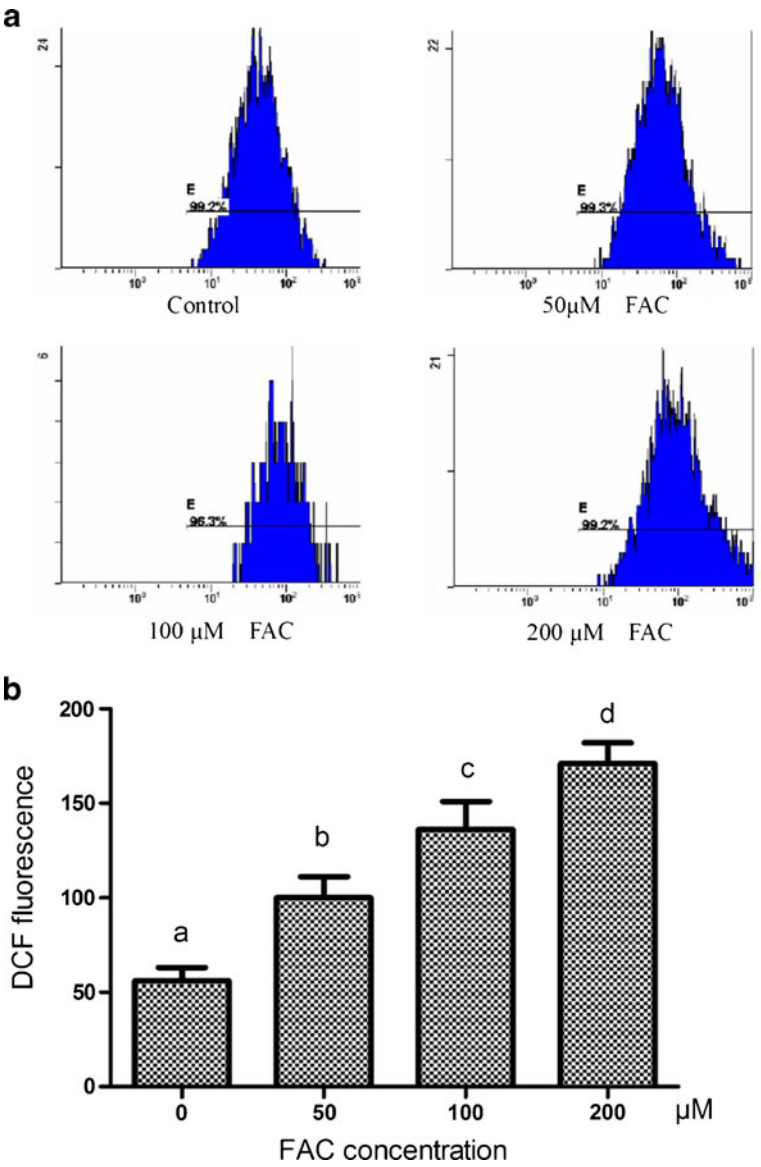

Fig. 8 Intracellular ROS levels of osteoblast after exposure to FAC at various concentrations. **a** The flow cytometric analysis of the osteoblasts treated with FAC at various concentrations. **b** DCF fluorescence representing intracellular ROS was increased with the increasing of FAC in a concentration-dependent manner. The results were expressed as the means±SD. N=3 wells per treatment per independent study. *Means with different letters* were significantly different (p<0.05)

FAC, a complex salt composed of iron, ammonia, and citric acid, is an ideal donor of ferric ion. The bioavailability of FAC is better than ferrous sulfate. In this study, we found that FAC treatment inhibited biological activities of hFOB1.19 cells along with increased intracellular ROS. Our findings are consistent with the widely held view that free iron can damage cells by producing ROS. ROS can cause lipid peroxidation, changes in cell membrane composition and fluidity, and alteration of proteins and DNA [24, 25]. In addition, it was recently reported that ROS could inhibit differentiation of osteoblast MC_3T_3-E_1 [26, 27]. It seems to be possible that the excessive iron inhibits osteoblast hFOB1.19 metabolism through increased intracellular ROS.

To investigate the effect of low iron status on osteoblasts, iron in culture media was chelated using DFO. DFO is an effective iron chelator and could decrease free iron concentration [28]. Our study showed that at low concentration of 5 or 10 μmol/L, DFO is conducive to the proliferation, differentiation, and mineralization function of osteoblasts and reduces apoptosis of the cells. Our data seem to be consistent with those of Zarjou et al. [29], who reported that DFO at 10 μmol/L can promote ALP activity and expression of OC. Moreover, similar results were reported with lactoferrin, a natural iron-scavenging protein [30]. Recently, it also has been found that oral iron chelators can improve osteoporosis in ovariectomized rats, which has been proven to be related to the reduction of iron overload in the body [31, 32]. These studies indicate that mild low iron is beneficial to osteoblast metabolism.

Iron deficiency or a decrease in its availability inhibits cellular DNA synthesis by decreased activity of ribonucleotide reductase enzyme [33, 34]. Consistent with this view, we demonstrated that at a high concentration of 20 μmol/L, DFO partly inhibited osteoblast activity. Qu et al. [35]

reported that ALP activity in $hBMSC_S$ was not decreased until DFO level was increased to 30 μmol/L. Our data were not completely consistent with the observation of Qu et al. This difference may be attributed to a number of reasons, including the use of hFOB1.19 (with relatively low osteogenesis activity in the current study vs. BMP2-treated MSC_S in the previous studies). The biphasic effects of DFO indicate that osteoblast metabolism needs iron concentrations in an appropriate range. Both iron excess and deficiency are harmful to osteoblast biological activities.

In the study, the expression of COL-I and OC did not display a biphasic response to iron depletion. This observation may reflect relatively narrow DFO concentrations used in the current study, as opposed to wider DFO concentration range (5–80 μmol/L) in previous studies that showed biphasic effects on the expression of OC of MG-63 cells [36]. In this study, we do not examine the iron concentration in the culture medium. In addition, bone homeostasis is maintained by the balance between osteoblastic bone formation and osteoclastic bone resorption. Iron has been shown to promote osteoclast activity [15, 37], but the specific dose–effect relationship was not further evaluated, which remains to be further studied.

In conclusion, excess iron inhibited osteoblast metabolism in a concentration-dependent manner. Mild low iron promoted osteoblast activity, while severely low iron inhibited osteoblast activity. Osteogenesis was optimal in certain iron concentration range. The mechanism underlying biological activities invoked by excessive iron may attribute to increased intracellular ROS levels. These findings indicated a useful strategy for the prevention and treatment of iron-related bone diseases.

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