Effects of Iron Overload on the Bone Marrow Microenvironment in Mice

Yuchen Zhang1, Wenjing Zhai2, Mingfeng Zhao1,*, Deguan Li3, Xiao Chai1, Xiaoli Cao1, Juanxia Meng1, Jie Chen1, Xia Xiao1, Qing Li1, Juan Mu1, Jichun Shen4, Aimin Meng3

1 Department of Hematology, Tianjin First Central Hospital, Tianjin, China, 2 Department of Stem Cells Transplantation, Blood Disease Hospital of Chinese Academy of Medical Sciences, Tianjin, China, 3 Key Lab of Radiation Medicine and Molecular Nuclear Medicine, Institute of Radiation Medicine, Academy of Medical Science and Peking Union Medical College, Tianjin, China, 4 Department of Hematology, Affiliated Hospital of Logistics University of People’s Armed Police Force, Tianjin, China

☯ These authors contributed equally to this work.
¤ Current Address: Department of Hematology, Tianjin First Central Hospital, No. 24 Fu Kang Road, Tianjin, China
* zmfzmf@hotmail.com

Abstract

Objective

Using a mouse model, Iron Overload (IO) induced bone marrow microenvironment injury was investigated, focusing on the involvement of reactive oxygen species (ROS).

Methods

Mice were intraperitoneally injected with iron dextran (12.5, 25, or 50mg) every three days for two, four, and six week durations. Deferasirox (DFX) 125mg/ml and N-acetyl-L-cysteine (NAC) 40mM were co-administered. Then, bone marrow derived mesenchymal stem cells (BM-MSCs) were isolated and assessed for proliferation and differentiation ability, as well as related gene changes. Immunohistochemical analysis assessed the expression of haematopoietic chemokines. Supporting functions of BM-MSCs were studied by co-culture system.

Results

In IO condition (25mg/ml for 4 weeks), BM-MSCs exhibited proliferation deficiencies and unbalanced osteogenic/adipogenic differentiation. The IO BM-MSCs showed a longer double time (2.07 ± 0.14 days) than control (1.03 ± 0.07 days) (P < 0.05). The immunohistochemical analysis demonstrated that chemokine stromal cell-derived factor-1, stem cell factor -1, and vascular endothelial growth factor-1 expression were decreased. The co-cultured system demonstrated that bone marrow mononuclear cells (BMMNCs) co-cultured with IO BM-MSCs had decreased colony forming unit (CFU) count (p < 0.01), which indicates IO could lead to decreased hematopoietic supporting functions of BM-MSCs. This effect was associated with elevated phosphatidylinositol 3 kinase (PI3K) and reduced of Forkhead box protein O3 (FOXO3) mRNA expression, which could induce the generation of ROS.
Results also demonstrated that NAC or DFX treatment could partially attenuate cell injury and inhibit signaling pathway triggered by IO.

**Conclusion**

These results demonstrated that IO can impair the bone marrow microenvironment, including the quantity and quality of BM-MSCs.

**Introduction**

Iron overload (IO) is a disease characterized by excessive iron deposition in tissues and damage to vital organs including heart, liver, and kidney. It can be caused by hereditary hemochromatosis or repeated blood transfusions for diseases such as beta thalassemia, bone marrow failure, or myelodysplastic syndrome [1–3]. Excess iron in the human body can lead to toxic effects such as cardiomyopathy, hepatic fibrosis, glucose intolerance, impotence, arthropathy, and even hematological disorders. Increasing clinical evidence has proven that iron chelation therapy can improve hematological parameters and reduce transfusion requirements [4–5], indicating that IO has a suppressive effect on hematopoiesis.

Bone marrow-derived mesenchymal stem cells (BM-MSCs) which are located in the hematopoietic niche, have been assumed to be a precursor cell with their further differentiated progeny constituting the functional components of the bone marrow microenvironment that supports hematopoiesis via secretion of cellular factors and maintaining the stability of the hematopoietic microenvironment [6–10]. Previous studies have shown that a deficiency of myeloid and erythroid cells in IO patients could be caused by damage to hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs) [11]. Based on this, it is reasonable to assume that primary BM-MSCs damage might exist in IO diseases, and increasing data has indicated that reactive oxygen species (ROS) are involved in the pathology of IO in vitro. Preliminary research revealed that under iron overload conditions, MSCs were deficient in proliferation and exhibited increased apoptosis. This progressed relative to catalyzing oxidative stress, and there was a positive correlation between ROS levels and labile iron pool (LIP) [12, 13]. It has been known that the effect of IO on hematopoietic stem/progenitor cells involved cellular senescence and apoptosis by up-regulating ROS level [14, 15]. However, the mechanisms involved in bone marrow (BM) microenvironment injury have not been clearly defined, and it is important to evaluate whether IO induces deficiency in BM microenvironment, particularly at the cellular and molecular levels.

This study established IO, iron-chelation, and anti-oxidative mouse models, then investigated general characteristics of BM-MSCs such as proliferation, osteogenic/adipogenic differentiation potential, and hematopoiesis supporting capacity. Finally, the related signal pathway in this process were investigated.

**Materials and Methods**

**Animal and treatment**

C57BL/6-Ly-5.1 (Ly5.1) male mice were obtained from the Institute of Peking university health science center (Beijing, China). The mice were bred at the certified animal care facility in the Institute of Radiation Medicine of PUMC (Peking Union Medical College). All mice were used at approximately 6–8 weeks of age, and the average weight was (20±0.24 mg). The Institutional
Committee of Animal Care and Use of PUMC approved all experimental procedures of our study. First, the IO mouse model was established by intraperitoneal injection of varying doses (12.5, 25, or 50 mg) of iron dextran in 1 ml saline every three days for durations of two, four, and six weeks.

Then, mice were randomly divided into four groups: control group, IO group (25mg/ml), Fe+Deferasirox (DFX) group, and Fe+N-acetyl-L-cysteine (NAC) group. The non-control groups were intraperitoneally injected with 25mg of iron dextran (Pharmacocmos) eight times over four weeks. The control group received an intraperitoneal injection with isovolumic saline. The DFX powder (Novartis, Basel, Switzerland) was suspended in 0.5% aqueous Klucel hydroxypropylcellulose by ultrasonication, and each mouse received 125mg/kg by gavage for 5 days a week. For NAC (Sigma Aldrich, St. Louis, MO USA) treatment, mice were given NAC in their drinking water (40 mM). The water bottles were exchanged twice weekly with a freshly prepared NAC solution [15]. All treatments lasted four weeks. Experimental animal treatment met local regulations and ethical requirements.

**Isolation and culture of BM-MSCs**

BM-MSCs were isolated as described previously [16]. Mice were killed by cervical dislocation and then liberally rinsed in a beaker with 100 ml of 70% (vol/vol) ethanol for 3 min. An incision was made through the skin and the muscles were dissociated. The muscles and tendons were then cleaned from humeri, tibiae, and femurs. Removal of the epiphyses was performed with sterile scissors. The bone marrow was flushed with a syringe needle with 3ml of $\alpha$-MEM (GIBCO, CA, USA) thoroughly, and then the compact bone was excised into 1–3 mm$^3$ sections with scissors and transferred into a 25-cm$^2$ plastic culture flask with forceps. The chips were digested for 1h in a shaking incubator at 37°C. When the bone chips became loosely attached to each other, they were aspirated and the digestion medium and released cells were discarded. The bone chips were washed and seeded them into a plastic culture flask in the presence of 5 ml $\alpha$-MEM supplemented with 10% (vol/vol) FBS (GIBCO, CA, USA) in the presence of 1mg/ml of collagens II(GIBCO, CA, USA). The chips digested for 1h in a shaking incubator at 37°C. When the bone chips became loosely attached to each other, they were aspirated and the digestion medium and released cells were discarded. The bone chips were washed and seeded them into a plastic culture flask in the presence of 5 ml $\alpha$-MEM supplemented with 10% (vol/vol) FBS and incubated at 37°C in a 5% CO2 incubator for 3 days. Then, the culture medium was changed. After 7d in culture, the adherent cells were harvested by removing the medium and adding 3 ml of 0.25% (mass/vol) trypsin/0.02% (mass/vol) EDTA (GIBCO, CA, USA) and run past the cells. Passage 1(P1) cells were prepared for the following experiment.

After isolating the BM-MSCs and P1, this cell population was comprised of morphologically homogenous fibroblast-like cells. PI staining showed that the viability of the cells was >95%. The regular marker of BM-MSCs was identified using flow cytometry, and FACS analysis showed that these cells were homogenously positive for the mesenchymal markers CD29 (integrin), CD44 (receptor for hyaluronate and osteopontin), CD105 (endoglin), and the stem cell marker Sca-1. It was negative for hematopoietic markers CD11b, CD34, CD45, endothelial cell marker CD31, and the costimulating molecule CD86. The cell surface antigen profile showed that the cells cultured were BM-MSCs.

**Identification of IO mice model**

The deposition of iron into the liver, spleen, bone marrow, and BM-MSCs was assessed using hematoxylin and eosin (HE) staining and Perls’ iron staining. Additionally, LIP level of bone marrow mononuclear cells (BMMNCs) and BM-MSCs was measured by Calcein-AM fluorescent Dye (Sigma) [17]. The P1 BM-MSCs were washed twice in PBS, then incubated (5x10$^5$ cells/well) for 15 minutes at 37°C with 0.125 μM Calcein-AM, and analyzed by a flow cytometer with the mean fluorescence intensity (MFI) calculated by the Cell Quest software.
Assess the proliferation ability of BM-MSCs

First, cells were plated in six well plates (1×10^5 cells/well) in growth medium with the medium replaced every third day. When cell confluency was 80%–90%, cells were digested at 37°C for 3 min with 0.25% trypsin, then counted and passed. Population doubling time (DT) was calculated with the following equation (10): DT = CT × log2 /log (X1 /X0), where CT is culture time, X1 is cells after confluence, and X0 represents the number of cells before seeding. Cell counting kit 8 (CCK8) (Bei Bo, Shanghai, China) is a compound similar to MTT, which was used to evaluate the cell proliferation ability. Cells were seeded at a density of 1×10^4 /well in 96-well plates for 48h, then mixed with 8ul of CCK8 solution/well and incubated for 3 hours at 37°C. The amount of formazan dye generated by cellular dehydrogenase activity was measured for absorbance at 450 nm with a microplate reader. The optical density (OD) values of each well represented the survival/proliferation of BM-MSCs. (Bio-Rad, Laboratories, Richmond, CA).

Differentiation induction

For Osteogenic induction, cells were plated at a density of 5×10^4 cells/well in a 24-well plate cultured in α-MEM supplemented with 10% (vol/vol) FBS, 10^-6 M dexamethasone, 0.5 μM IBMX, and 10 ng/ml (mass/vol) insulin according to a previous study [16]. Alkaline phosphatase (ALP) has been used as a routine marker for osteogenic differentiation of BM-MSCs [18, 19]. Intracellular ALP activity normally increases readily during in vitro osteogenesis and reaches a peak after 2 weeks. ALP activity can be assessed using an Alkaline Phosphatase Kit (Sigma-Aldrich, St. Louis, MO USA) following the manufacturer’s instructions. Furthermore, in order to assess the formation of mineralization nodule after 4 weeks of induction, culture plates were rinsed with phosphate buffered saline (PBS), fixed with 4 paraformaldehyde, and stained with 2% Alizarin red S (pH 4.0) (Sigma-Aldrich, St. Louis, MO USA).

The adipogenic differentiation ability of BM-MSCs was determined by oil red staining (ORO) (Sigma-Aldrich, St. Louis, MO USA). Cells were seeded in 24-well plates at 2×10^5 cells/well with growth medium, when the cell confluence reached 70%–80%, the medium was changed to adipogenic differentiation medium (GIBCO, CA, USA). The media was changed every third day. On day 14, cells were washed with PBS twice, held for 30 minutes at room temperature with 10% formaldehyde, stained with freshly diluted ORO solution for 15 minutes. Then, the plates were washed with distilled water three times, and observed under the inverted phase contrast microscope reader.

Analysis of hematopoietic stem cells (HSC) and hematopoietic progenitor cells (HPC) by Flow Cytometry and establishment of co-culture system

BMMNCs were incubated with biotin-conjugated rat antibodies specific for murine CD5, Mac-1, CD45R/B220, Ter-119, and Gr-1 for 15 min at room temperature. After washing with PBS twice, the cells were stained with APC-Cy7-conjugated Streptavidin, PE-Cy7-conjugated anti-Sca1, and Alexa Fluor 700-conjugated anti-c-kit antibodies (eBioscience, San Diego, CA, USA) and analyzed by flow cytometry. The ratio of HPC (hematopoietic progenitor cells, Lin^− c-kit^+ Sca-1^−) and HSC (hematopoietic stem cells, Lin^− c-kit^+ Sca-1^+) in BMMNCs was calculated.

The co-culture system was established as described previously [20, 21]. BM-MSCs were treated with misogynic C (200ug/ml) for 2h to prevent excess proliferation and deterioration of culture conditions, and were then immediately plated at a density of 1×10^5 cells/well in 2.5 mL long-term bone marrow cultures (MyeloCult H5100, Stem Cell Technology) medium in...
collagen-coated 6-well plates (collagen from rat tail, Gibco). At 24 h after misogynic C treatment, \(5 \times 10^5\) BMMNCs were seeded in each well and incubated at 37°C in a humidified incubator with 5% CO2 in air without changing the medium. After one week, the suspended BMMNCs were removed from the co-culture system and subjected to Colony-forming cell (CFC) assay in MethoCult GF M3434 methylcellulose medium (Stem Cell Technologies, Vancouver, BC). \(2 \times 10^5\) BMMNCs were added to 2ml M3434 medium tube and vortexed. After allowing the bubbles to dissipate, cells were dispensed into pre-tested culture dishes using a syringe with blunt-end needle. The cells were incubated for 5–14 days in a humidified incubator at 37°C and 5% CO2. Colony-forming unit erythroid (CFU-E), burst-forming unit erythroid (BFU-E), colony-forming unit granulocyte-macrophage (CFU-GM), and colony-forming unit mix (CFU-Mix) were counted on days 5, 7, 9 and 12 respectively, using a microscope according to the manufacturer’s protocol.

**Immunohistochemistry of mouse bone marrow sections**

Immunohistochemical staining was performed on BM biopsies. All samples were fixed in formalin mixture, decayed in EDTA, and embedded in paraffin. Serial sections 3μm thick were processed for immunohistochemical staining with antibodies CXCL12, SCF-1, and VEGF-1 (Abcam, USA).

**Analysis of the levels of intracellular ROS**

BM-MSCs were incubated with DCFH-DA 10 μM (Sigma-Aldrich) in a humidified atmosphere of 5% CO2 at 37°C for 15 minutes, and the levels of intracellular ROS were analyzed by measuring the mean fluorescence intensity (MFI) of 2’-7’ dichlorofluorescein (DCF) using a flow cytometer.

● **Quantitative real-time polymerase chain reaction (qRT-PCR) analysis**

Then, the PI3K, FOXO3 mRNA expression of BM-MSCs were analyzed, which are related to the elevation of ROS.

Osteogenic and adipogenic differential genes including ALP, runt-related transcription factor 2 (RUNX2), osteocalcin (OCN), collagen II (COL II), peroxisome proliferator activated receptor gamma (PPARg), adipocyte fatty acid binding protein (ap2), and Adipsin were detected by quantitative real-time RT-PCR assays. The primer sequences are listed in Table 1. Total RNA was isolated from BM-MSCs using the TRizol reagent (Sigma-Aldrich) following the manufacturer’s protocol. First-strand cDNA was synthesized from total RNA using an RNA PCR kit (AWV) version 3.0 (Takara, Japan) according to the manufacturer’s protocol. PCR primers were obtained from Sangon Biotech (Shanghai, China). The cycle threshold values were normalized to the expression of the housekeeping gene β-actin. The changes in target gene expression were calculated by the comparative \(C_T\) method (fold changes = \(2^{-\Delta\Delta C_T}\)) as previously described and calculated [22].

**Statistical Analysis**

All experiments were performed at least three times. The results are presented as mean ± standard deviation (SD). Multiple group comparisons were performed using analysis of variance (ANOVA). Differences were considered to be statistically significant at \(p < 0.05\). All of the analyses were performed with the GraphPad Prism program (GraphPad Software, Inc. San Diego, CA).
Table 1. List of the primers used in this study.

| Gene   | forward                  | reverse                  |
|--------|--------------------------|--------------------------|
| RUNX2  | GAGGTACCCAGAT GGGACTGTG  | TCGTTGAACTTGTCATTTGG     |
| ALP    | TCGGGACTGTGACTCGGATAAA   | CTGGTAGCTTGAGGCTGAAAT    |
| OCN    | CCTGCTCTCTGACCTCACAG     | GGAAGCTGTGTGACATCCATA    |
| COL    | GAGGCATTAAGGGTGATCGTG    | CATAGGCGCGAAGGTGCAGC     |
| FOXO3  | TTAGAGGCGATGCCAATAAA     | GCGGCACAGTATTTCTGA       |
| PPARg  | CGCTGATGCACCTGCTATGA     | GGGCCAGATGGCGATCTCT      |
| Adipsin| TGGTGAGTGAAGCAGTGGGT     | AAGGTTACAGACGTCAGGAGG    |
| aP2    | GCCTAGAAAGGGACTGGTC      | TTCTGGATCAGTGCTTGATT     |
| PI3K   | ACTTGTAGCCTAGGCTTTT      | TACTTCTGACATCTCTCTC      |
| VEGF   | GCCTAGAATCCGCTGACGCTATGG| ATGATGCTGACTCAGCCGCC     |
| CXCL12 | CCGGCATCATGAACGCAAGGCTGTG| AGAGCTGGGCCTACTGTCGGCGGGC|
| SCF    | TTATGTACCCCCCTGTTGAGC   | CGGCCCTGATAGACTGACTG     |
| AKT    | TGCTATTGAGAATGTCGCGTCTC | AGGCTAACCAGGAGGTAAGA     |
| β-actin| ACGGCCAGGTCTACACATTTG    | CGCTTTGCTGATCCACATCT    |

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Ethics Statement

This study has been approved by the Ethics Committee of Peking Union Medical College (No. 2012–0504).

Evaluation of the benefits for the experimental animals: (1) The experiment, in which the needs of the animals were fully considered, was approved by the ethics committee, including physiological (adequate food, water, temperature, and illumination), environmental, psychological, and social needs (socially raised, 4–6 animals per cage, avoiding fatigue and overstimulation). The outcomes of the preliminary experiment and the literatures were taken into consideration to make rational design of the sample size and operation standard. (2) A daily observation was performed to prevent the animals from anger, comfortlessness, fear, nervousness, pain or damage; and to keep them at baseline status. Abuse and excessive or incorrect medication was avoided. For subcutaneous injection, narcotics were not provided. For tail vein injection, intraperitoneal anesthesia was given to alleviate pain. (3) At the time of endpoint, the animals were slaughtered within 15 s to avoid nervousness of the other animals.

Results

The establishment of IO mouse model

To confirm the efficacy of the IO mouse model, the LIP levels of the BMMNCs were evaluated. The results showed that the LIPs of the BMMNCs gradually increased in a time and dose-dependent manner. When the mice were injected with 25mg/ml iron dextran for 4 weeks, the LIP level of BM-MSCs in the IO group increased and maintained at a stable level, and could be reversed following administration of DFX or NAC (Fig. 1A). Furthermore, the hepatic, splenic, and BM iron deposits were assessed in the end of fourth week. Iron deposits were easily observable in the liver, spleen, BM, and BM-MSCs (Fig. 1B–C). These results demonstrated that this experimental murine model reflected an iron-overloaded pathogenic condition.

IO inhibited BM-MSCs proliferation ability

In preliminary study, it was found that exposure of mice to varying doses (12.5, 25, or 50mg/ml) of iron dextran reduced the number of BM-MNCs and frequency of CFUs in a dose and
Fig 1. The establishment of an IO mouse model. (A) The LIP level of BM-MSCs in the condition of 25mg/ml iron dextran for 8 time points. (B) The hepatic, splenetic and BM iron deposits were exposed to hematoxylin-eosin staining (x400). (C) BM and BM-MSCs were subjected to Perl's iron staining (x1000).

***P < 0.001 compared with control group, ###P < 0.01 compared with Fe group, ##P < 0.05 compared with Fe group).
time-dependent manner. To further characterize IO-induced BM microenvironment injury, mice were injected with 25mg/ml iron dextran for 4 weeks, and BM-MSCs were isolated. The cell viability and proliferation ability were evaluated by CCK8 assay and DT. As shown in Fig. 2, under IO condition, the cell viability was diminished, and the DT of BM-MSCs was (2.07±0.14) d, which was significantly longer than that of control (1.03±0.07) d (P<0.05). Both effects could be partly restored by treatment with DFX (1.52±0.07) d or NAC (1.68±0.03) d.

IO inhibited osteogenic differentiation and increased adipogenic differentiation of BM-MSCs

To confirm the effect of IO on BM-MSCs osteogenic differentiation and mineralization, cells were isolated and cultured in induction medium for 14 days. Compared with the control group, the expression of Alkaline Phosphatase (ALP) in the IO group was decreased (Fig. 3A). After 4 weeks of induction, the mineralized nodules formed in the IO group were less than that of the control group (Fig. 3B). In order to investigate the effect of IO on lipid droplet formation of lipoblast in each group, cells were fixed and stained with ORO following culture in induction medium. The results indicated that lipid accumulation significantly increased in the IO group compared with that of control (Fig. 3C). After co-administration with DFX and NAC, this effect could be partly alleviated.

IO inhibited expression of SCF, CXCL12, and VEGF

Next, the expression of hematopoiesis chemokines and adhesion molecules in bone marrow was investigated. Immunohistochemical analysis demonstrated that the levels of SCF, CXCL12, and VEGF were lower in bone marrow sections from IO mice compared to those from control mice (Fig. 4), suggesting that IO bone marrow results in down-regulated expression of these hematopoietic factors. Moreover, DFX and NAC treated IO mice showed increased expression of all three factors above, which indicates that IO injury effects could be alleviated by iron-chelation and anti-oxidative therapy.
Fig 3. IO inhibited BM-MSCs osteogenic differentiation. (A) Osteogenic differentiation was assayed with in situ alkaline phosphatase staining (×400), ALP score of each group (p<0.05). (B) Evaluation of mineralized nodules by alizarin red S staining after 4 weeks induction (×100). Fig(C) Adipogenesis of BM-MSCs was stained with Oil-Red-O. IO increased adipogenic differentiation of BM-MSCs compared with the control group, which could be reversed by DFX and NAC (×400). (**P<0.001 compared with control group, *P<0.05 compared with control group, ###P<0.001 compared with Fe group, ##P<0.01 compared with Fe group).

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IO decreased the ratio of HPC and HSC in BMMNCs, and impaired the supporting function of BM-MSCs

Cell dysfunction is considered to be a feature of oxidative stress. Pancytopenia as a result of excessive iron-induced oxidative stress also revealed prominent reductions in colony-forming capacity of BMMNCs [13]. It was examined whether IO could affect the quantity of hematopoietic cells in vivo. The ratios of HPC and HSC in BMMNCs was analyzed by FACS, and it was found that IO decreased the ratios of HPC and HSC in BMMNCs. Notably, this effect could be reversed after iron-overloaded mice were administered DFX and NAC (Fig. 5A).

Furthermore, to ascertain whether impaired hematopoiesis occurred indirectly as a result of decreased hematopoietic supportive capacity of MSCs, a co-culture system was performed using BM-MSCs, which supports HSC development. BMMNCs were co-cultured with iron-overloaded BM-MSCs for 7 days and then subjected to a CFC assay. Cells obtained at 1 week after co-culture with iron-overloaded BM-MSCs showed an expected decrease in CFU (P < 0.05, Fig. 5B).

IO increased the expression of ROS and activated the PI3K/FOXO3 pathway

It is known that iron is the main catalyst of ROS in an organism, and a growing body of evidence demonstrates that there is a positive correlation between ROS levels and LIP [12, 13]. This finding prompted examination of whether IO induced oxidative stress in BM-MSCs in vivo. As shown in Fig. 6, there was a significant increase in ROS levels in the IO group compared to the control group, which could be reversed by DFX or NAC treatment (P < 0.001). In order to provide further mechanistic evidence for the role of oxidative stress in this progress, the expression of PI3K and FOXO3, which may mediate IO-induced ROS production, were
Fig 5. IO decreased the ratio of HPC and HSC in BMMNCs and impaired the supporting function of BM-MSCs. (A) Iron overload decreased the ratio of HPC and HSC in BMMNCs, which could be reversed by DFX or NAC. (B) Iron overload impaired the supporting function of BM-MSCs. The colony-forming cell (CFC) assays (CFU-GM, CFU-E, BFU-E, and CFU-mix) were performed as means±SE of three independent experiments. (**P<0.001 compared with control group, ###P<0.001 compared with Fe group, ##P<0.01 compared with Fe group, #P<0.05 compared with Fe group).

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quantified in BM-MSCs by real time RT-PCR. It was found that in the IO microenvironment, the expression of FOXO3 was down-regulated in BM-MSCs, whereas the expression of PI3K was elevated. This finding suggested that the activation of PI3K/FOXO3 signal pathway may mediate sustained increases in ROS production in IO BM-MSCs.

**IO inhibited the osteogenic differentiation gene expression and increased adipogenic differentiation gene expression of BM-MSCs**

In order to investigate the mechanism of excessive iron in BM-MSC osteoblastic/adipogenic differentiation, osteogenic differentiation gene expression (ALP, RUNX2, OCN) and adipogenic differentiation gene expression (PPARg, adipsin, aP2) of BM-MSCs in each group was analyzed. Compared to control values, a significant decrease in RUNX2, ALP, and OCN mRNA expression was observed ($P < 0.05$) (Fig. 7A). These changes could be reversed by iron-chelation and anti-ROS therapy. At the same time, IO induced ROS increased expression of adipogenic genes. The mRNA levels of the adiponectin differentiation markers PPARg, adipsin, and aP2 (Fig. 7B) were increased in BM-MSCs under IO conditions, but prevented by DFX and NAC.

**IO reduced mRNA expression of SCF, VEGF, and CXCL12**

Similar to the immunohistochemical analysis, notable changes in the expression of common haematopoietic cytokines, chemokines, and adhesion genes (such as SCF, VEGF, and CXCL12) were identified in the iron-overloaded BM-MSCs, all of which are key molecules for hematopoiesis (Fig. 8).

**Discussion**

Clinically, patients who receive regulated red blood cell transfusions may experience IO, which damages hematological function. Recently, it was reported IO has a suppressive effect on hematopoiesis in patients with MDS [23–25]. Iron chelation therapy improved the hematological parameters and decreased transfusion requirements in these patients, and this provided possible evidence supporting the toxic effect of free iron on hematopoiesis. Okabe et al. also found that IO can significantly delay hematopoietic recovery after bone marrow transplantation, which indicates that IO impacts the hematopoietic microenvironment of BM [26]. However, the exact mechanism still needed to be determined. This study first established an IO, iron—chelation, and anti-oxidative mouse model. The result showed significantly deficient
hematopoiesis under IO conditions, which was caused mainly due to microenvironment injury; especially to the BM-MSCs.

An IO mouse model was established by injecting different concentrations of iron dextran (12.5, 25, or 50mg/ml) for varying durations of time (2, 4, or 6 weeks) to confirm the inhibitory effects caused by IO. The morphologies of the liver, spleen, and bone marrow has clearly demonstrated that iron deposition. BM-MSCs were then isolated; and the results showed that in the IO group, iron deposits were apparent. After treatment with DFX, the iron deposits and LIP levels decreased, which validated the IO animal model.

Fig 7. IO induced ROS inhibited osteogenic genes expression and increased adipogenic genes expression in BM-MSCs. Real-time PCR was performed to quantify the expression of osteogenic genes, such as (A) Runx2, ALP, collagen I, and adipogenic genes and (B) PPARg, aP2, and adipin. All expression of target genes was normalized to β-actin gene expression. (***P<0.001 compared with control group, *P<0.05 compared with control group, ###P<0.001 compared with Fe group, ##P<0.01 compared with Fe group)

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Fig 8. IO induced ROS inhibited haematopoietic chemokines and adhesion molecules genes expression in BM-MSCs. Real-time PCR was performed to quantify the expression of haematopoietic chemokines and adhesion molecules. Genes related to haematopoiesis (chemokines, cytokines, and adhesion molecules) that were suppressed in IO BM-MSCs. (***P<0.001 compared with control group, ###P<0.001 compared with Fe group).

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A previous study demonstrated that IO had negative effects on hematopoiesis [11, 26]. However, the exact mechanism involved in this process still needed to be clarified. After establishing the IO model system, an assessment was made of whether IO injured the proliferation potential of BM-MSCs. Results showed that in the IO group, the doubling time of BM-MSCs was decreased but could be reversed by DFX and NAC. Increased cellular LIP could enhance the production of ROS, which would stimulate a series of cell signaling pathways to induce cell apoptosis and block the cell cycle into G0/G1 phase, resulting in a decreased proliferation [27–29].

The study also found that the osteoblastic/adipocytic balance of BM-MSCs was broken by IO. Calcium deposition was used as a marker for osteogenic differentiation [30] since cells with an osteoblastic phenotype deposit increased amounts of extracellular matrix, which mineralizes over time. In IO conditions, the ALP expression and mineralized nodule formation of osteoblasts was significantly decreased. The possible mechanism might be associated with a decreased expression of ALP, RUNX2, and OCN; which are common osteoblastic differentiation markers [31]. Osteoblasts were identified as crucial components of the hematopoietic supportive stroma, which is important for hematopoietic stem cell proliferation and mobilization [32, 33]. However, Oil Red O (ORO) staining indicated that lipid accumulation significantly increased in IO group lipoblasts compared with that of control group. These observations suggested a disturbance in the osteoblast/adipocyte balance. Some cases of such imbalances have already been reported in stress conditions as well as in pathological conditions, such as hyperglycemia and hypoxia [34, 35]. Additionally, patients with thalassaemia and MDS, who often received repeated blood transfusions, also showed lower alkaline phosphatase activity, lower gene expression of osteogenic differentiation markers, and develop progressive osteoporosis [36, 37]. These discoveries were consistent with other studies that have demonstrated that oxidative stress impairs osteoblast formation and function, as well as mineralization. [38]

ROS were generated via NADPH oxidase and the mitochondria electron transport chain [39]. Under normal condition, ROS is maintained at low levels by enzyme systems participating in the redox homeostasis. However, excessive accumulation of ROS might cause oxidative damage to proteins, membranes, and genes; which can lead to senescence and apoptosis of cells [40]. In this study, it was found that there was a positive correlation between LIP and ROS level, which may relate to the activation of the PI3K/AKT/FOXO3 signal pathway. PI3K/AKT has been recognized as a vital pathway that up-regulates intracellular ROS levels [41]. AKT promotes metabolic activity in the mitochondria and inhibits FOXO transcriptional activity, leading to high level of ROS. FOXO3 proteins function as key downstream affectors of growth-factor receptors, which participate in the regulation of many cellular processes including cell proliferation, apoptosis, longevity, and cell cycle [42]. As the pathway for maintaining ROS at normal levels, FOXO3 is essential for regulation of HSC [43]. Also, some scholars [44] have reported that chronic iron overload (CIO) can enhance inducible nitric oxide synthase (iNOS) expression in rat livers via extracellular signal-regulated kinase (ERK1/2) and NF-kappaB activation, triggering liver oxidative stress, which has a congenerous function with ROS. To resolve this issue, the focus will shift to changes of Reactive Nitric Species (RNS) level, such as nitric oxide, in following studies.

Immunohistochemical analysis revealed that IO inhibited the expression of CXCL12, SCF, and VEGF; which are vital molecule for hematopoiesis. In mouse bone marrow, CXCL12 mediated the arrest and adhesion of transplanted human hematopoietic progenitors [45] and circulating central memory mouse CD8+ T cells to the bone marrow endothelium. Hongting Jin et al. found that BM-MSCs can enhance new blood vessel growth both in vitro and in vivo [46]. In recent years, some scholars have suggested that transplantation of BM-MSCs enhances vascular regeneration, mainly through a paracrine action of VEGF, one of the most potent
mediators of angiogenesis [47]. These findings suggested that IO inhibited the expression of VEGF, and may damage the generation of sinus in bone marrow, which is vital for hematopoiesis. SCF is also a vital molecule associated with CD34+ cell proliferation. Reduced expression of these molecules in the bone marrow may be an important clue to the formation of defects in hematopoietic associated IO conditions.

In conclusion, evidence has been provided that increased ROS during IO injured the proliferation potential and disturbed the differentiation balance of BM-MSCs. Administration of DFX and NAC may lead to a reversal. Improving the function of BM-MSCs may serve as a new strategy to enhance normal hematopoietic in IO bone marrow.

Author Contributions
Conceived and designed the experiments: MFZ. Performed the experiments: YCZ WJZ. Analyzed the data: DGL XC JM. Contributed reagents/materials/analysis tools: XLC JXM. Wrote the paper: JC XX. Collating of data: JCS AMM QL.

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