Comparison of the effects of deferasirox, deferoxamine, and combination of deferasirox and deferoxamine on an aplastic anemia mouse model complicated with iron overload

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Background and aim: Iron overload is commonly observed during the course of aplastic anemia (AA), which is believed to aggravate hematopoiesis, cause multiple organ dysfunction, lead to disease progression, and impair quality of life. Deferasirox (DFX) and deferoxamine (DFO) are among the most common iron chelation agents available in the clinical setting. The aim of this study was to investigate if the combination therapy with DFX and DFO is superior in hematopoietic recovery and iron chelation.

Methods: Briefly, we developed a composite mouse model with AA and iron overload that was consequently treated with DFX, DFO, or with a combination of both agents. The changes in peripheral hemogram, marrow apoptosis, and its related protein expressions were compared during the process of iron chelation, while the iron depositions in liver and bone marrow and its regulator were also detected.

Results: The obtained results showed that compared to DFX, DFO has a better effect in protecting the bone marrow from apoptosis-induced failure. The combination of DFO and DFX accelerated the chelation of iron, while their efficiency on further hemogram improvement appeared limited.

Conclusion: To sum up, our data suggest that single treatment with DFO may be a better choice for improving the hematopoiesis during the gradual chelation treatment irrespective of the convenience of oral DFX, while the combination treatment should be considered for urgent reduction of the iron burden.

Keywords: aplastic anemia, iron overload, mouse, animal model, deferasirox, deferoxamine

Introduction
Aplastic anemia (AA) is a rare, but potentially life-threatening bone marrow failure disorder characterized by pancytopenia with an increased risk of hemorrhage and infection. In People’s Republic of China, the incidence of AA is nearly triple that in Western countries. Moreover, most of the cases fall under the nonsevere acquired AA category. Immune suppression therapy and stem cell transplantation are considered the first-line treatments for AA; nevertheless, not all patients can afford this therapy, or reach remission with it, and thus many patients still require blood transfusion as supportive therapy. Iron overload, which is transfusion-dependent or can be ascribed to the disease-related dysfunction in iron metabolism, is a common complication during the course of AA. As reported, patients who receive more than 20 U red blood cells via transfusion can develop iron overload. Iron chelation therapy, which is becoming increasingly popular in the treatment of bone marrow failure diseases, like...
myelodysplastic syndrome (MDS) and AA, is believed to be efficient in protecting organs (liver, heart, etc.), improving hematopoiesis, and promoting the quality of life.\(^6\) There are some clinically available iron chelation agents, such as deferasiprone,\(^7\) deferasirox (DFX),\(^9\) and deferoxamine (DFO),\(^1\) which are widely used in thalassemia. DFX and DFO are also the most commonly used agents for AA.\(^8\)–\(^10\) During treatment, those chelators should be continuously present in the circulation in order to achieve the best iron chelation effect. DFO should be continuously administered for at least 8–12 h subcutaneously, 5–7 days a week, which is not very convenient for outpatients, while DFX is an oral iron chelator and as such has a better patient compliance.\(^11\) The metabolisms of the 2 agents are different, and various factors may affect the efficacy of monotherapy, as well as the side effects.\(^12\) Nowadays, most patients with iron overload would prefer DFX to DFO, because of its oral administration. DFO, which has been proven to show iron-induced heart disease protection,\(^13\) may be indicated when DFX is not affordable or ineffective, especially in patients with severe iron overload or cardiac involvement. The combination therapy has been shown to be very effective in iron chelation for thalassemia,\(^7\)\(^14\) but there are no available data for AA with severe iron overload. Whether the combination treatment exerts a superior effect in iron deposition reduction and hematopoiesis recovery than the monotherapy remains unclear. In the present study, we compared the combination effect of iron chelation with DFX and DFO on an AA animal model complicated with iron overload, which was established by our group previously,\(^15\) as well as their effect on hematopoietic recovery, to clarify the underlying mechanisms.

**Materials and methods**

**Animals**

Forty clean-grade inbred female Balb/c mice aged 6–8 weeks, and 5 female DBA/2 mice aged 6–14 weeks were provided and fed by the Laboratory Animal Center of Zhejiang Chinese Medical University, Hangzhou, People’s Republic of China. All animals had free access to normal chow and water. The experiments were performed according to the National Institute of Health Guide for the Care and Use of Laboratory Animals (revised 1996\(^16\)) and were approved by the Animal Management and Ethics Committee of Zhejiang Chinese Medical University (No ZSLL-2013-108).

**Reagents**

The reagents used were as follows: Iron dextran (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany); DFX and DFO pure powders (Novartis Pharma Schweiz AG, Rotkreuz ZG, Switzerland); serum iron (SI) detection kit (cat. no A039-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, People’s Republic of China); serum ferritin (SF) enzyme-linked immunosorbent assay (ELISA) kit (cat. no E05187h, Cusabio Biotech Co., Ltd., Wuhan, People’s Republic of China); serum BMP-6 ELISA kit (cat. no CSB-E09277h, Cusabio Biotech Co., Ltd.); tissue hepcidin ELISA kit (cat. no CSB-E13062h, Cusabio Biotech Co., Ltd.); and TUNEL apoptosis assay kit (cat. no S7100, Millipore, Billerica, MA, USA).

**Animal grouping**

After the duplication of the composite model (AA complicated with iron overload), BALB/c mice (n=32) were randomly assigned into 4 groups and named as follows: DFX-treated group (DFX, n=8), DFO-treated group (DFO, n=8), DFO and DFX cotreated group (DFX + DFO, n=8), and composite model group (Model, n=8). Furthermore, 8 healthy BALB/c mice without the model duplication were considered as the normal group (Normal, n=8).

**Duplication of the AA mouse model complicated with iron overload**

The duplication of AA model complicated with iron overload was done according to our previous research.\(^15\) Briefly, Balb/C mice were first administered with iron dextran (200 mg/kg/wk, continue for 10 weeks) via intraperitoneal injection. After establishing the iron overload model, all the mice were treated with whole-body irradiation (\(^6\)Co 6.0 Gy at 1 Gy/min), following administration of 0.2 mL (5×10\(^6\) cells/mL) prepared thymus cell suspensions from DBA/2 mice; the injection was performed via the caudal vein within 4 h.

**Treatments**

DFX was given at a dosage of 0.2 g/kg (dissolved in normal saline) twice a day via abdominal subcutaneous injection, while DFX was given at a dosage of 0.2 g/kg (dissolved in distilled water) daily via intragastric administration for 5 weeks. The normal and model control groups received a corresponding volume of solvent by intragastric and abdominal subcutaneous administrations, respectively.

**Sample collection**

Blood was collected for routine testing from the orbital veins on days +14 and +35. Blood, liver, and bilateral femurs were extracted from randomly chosen mice (n=4) on day +35 following overnight fasting and euthanasia via cervical dislocation.
Detection of peripheral hemogram

Blood tests were performed using manual classification and counting protocols by the Department of Laboratory Medicine of Zhejiang Provincial Hospital of TCM (Hangzhou, People’s Republic of China). Briefly, the blood smears were prepared, stained with Wright–Giemsa stain, and observed under a light microscope (CX31RTSF, Olympus Optical Co., Ltd., Tokyo, Japan) at a magnification of 400×.

Pathomorphological observation

For all groups, the liver and unilateral femur were fixed in a 10% formalin solution for 24 h, and then the femur was decalcified by soaking in 5% nitric acid solution for 7–12 h. All of the tissues were dehydrated, paraffin-embedded, sliced up, stained with hematoxylin and eosin/iron (redyed by Sudan red), and consequently observed and analyzed under light microscopy at a magnification of 400×. The iron staining intensity was evaluated according to the extent of cell coloration: “−” represented negative staining; “+” represented mild staining with positively stained cells showed a light blue pigment; “++” represented moderate staining with positively stained cells showing a dusty blue pigment; and “+++” represented intense staining, with positively stained cells showing a dark blue pigment, with each scored based on 0, 1, 2, or 3 points, respectively. The total area occupied by iron deposition per 540,000 μm² of bone marrow area (excluding adipocytes) was evaluated using a Nikon Digital Image Analyzer using Imaging Software 3.10 (Nikon Corporation, Tokyo, Japan). The product of staining intensity and staining positive area ratio was further used for statistical analysis.

Detection of SI content

About 0.5 mL blood plasma was taken for the detection of SI content by absorption spectrophotometry using a microplate reader according to the SI detection kit instructions. The following equation was used:

\[
SI = \frac{Ad - Ab}{As - Ab} \times \text{standard concentration (35.81 μmol/L)}
\]

The absorbance of the detection tube (Ad), absorbance of the blank tube (Ab), and the absorbance of the standard tube (As) are the variables encountered.

ELISA

About 1–1.5 mL serum was taken from each group for the detection of SF and BMP-6, and 10% liver homogenate (0.5 mL) was prepared for the detection of liver hepcidin using the ELISA kit (Cusabio Biotech Co., Ltd) according to the manufacturer’s protocol.

TUNEL assay

Femur were fixed in a 10% formalin solution for 24 h, decalcified by soaking in 5% nitric acid solution for 7–12 h, and then paraffin-embedded and sectioned (thickness, 4 μm) before being HRP-conjugated with dUTP. TUNEL apoptosis assay kit was used according to the manufacturer protocol. The slides were observed under a fluorescence microscope (Olympus BX51T-PHD-J11, Tokyo, Japan). Counts of TUNEL-positive nuclei were performed by counting total number of stained nuclei across 400 μm sections of each sample.

Immunohistochemistry for apoptosis-related protein expression in bone marrow

The staining intensity was evaluated according to the extent of cell coloration: “−” represented negative staining; “+” represented mild staining with positively stained cells showing a yellow pigment; “++” represented moderate staining with positively stained cells showing a brown pigment; and “+++” represented intense staining with positively stained cells showing a dark brown pigment, each scored based on 0, 1, 2, or 3 points. The product of staining intensity and staining rate was further used for statistical analysis.

Western blot analysis

Cells were collected, washed 3 times with ice-cold PBS, and lysed on ice with lysis buffer (10 mM Tris pH 7.5, 130 mM NaCl, 1% NP-40, 10 mM NaP Pi, 1 mM PMSF, 0.1 mM Na4VO4). Lysates were transferred to microcentrifuge tubes and precleared by centrifugation at 11,900×g for 15 min at 4°C. Protein concentrations were determined using Bradford reagent according to the manufacturer’s instructions. β-actin was used as an internal reference control. An equal amount of total protein extracted from cultured cells was separated by 12% SDS-PAGE and transferred to PVDF membranes (EMD Millipore Corporation, Billerica, MA, USA). Primary antibodies and HRP-conjugated appropriate secondary antibodies were used to detect the designated proteins. The bound secondary antibodies on the PVDF membrane were reacted with the ECL detection reagents (Beyontime, Institute of Biotechnology, Jiangsu, People’s Republic of China) and exposed to X-ray films (Kodak, Tokyo, Japan). The result was analyzed using ImageJ 1.46r software (National Institutes of
Health, Bethesda, MD, USA). Anti-hepcidin, anti-BMP6, anti-SMAD4, anti-Tlr2 (Abcam, Eugene, OR, USA), anti-NF-κB (Protein Tech, Chicago, IL, USA), and β-actin monoclonal antibody (LiankeBio, Hangzhou, People’s Republic of China) were used in the experiments.

Statistical analysis
Data were collected and input into Excel and later analyzed by SPSS 17.0 (SPSS Inc., Chicago, IL, USA). All data were expressed as mean ± SEM. Analysis of variance was employed to compare the 2 groups of normally distributed data by using one-way analysis protocol and followed by a post hoc Newman–Keuls test. P<0.05 was considered to indicate statistically significant difference.

Results
DFO exerted a better effect on peripheral hemogram recovery
The completion of thymus cell suspensions from DBA/2 mice injected into model Balb/c mice was defined as day 0. To identify the successful duplication of AA mouse model complicated with iron overload, SI, SF, and hemogram were detected on day +14 and day +35. On day +14, the hemogram in AA mice reached the minimum value. On day +14, white blood cells (WBC), hemoglobin (Hb), and platelets (PLT) were significantly lower in the composite model group and all iron chelation group (DFX, DFO, and DFX + DFO) when compared to the normal group (P<0.01 or P<0.05), apart from Hb in DFO-treated group. Furthermore, both DFO and DFX increased the levels of WBC and Hb in the composite model mice; nonetheless, the significant difference was only observed in the DFO-treated group (P<0.01), while the effect was more obvious compared to DFX-treated group (P<0.01); In addition, DFX had a negative, but not significant, effect on the PLT count. Moreover, combined chelation treatment promoted the recovery of Hb (P<0.01), but no significant difference was observed when compared to other single chelation groups (Figure 1). On day +35, the WBC and PLT, but not Hb, were still significantly lower in the model control group, as well as in all chelation groups compared to the normal control group (P<0.01 or P<0.05). DFX had no significant effect on hemogram recovery when compared to the model control group, while DFO significantly increased the level of PLT (P<0.05); DFO had a better effect on WBC and PLT recovery than DFX (P<0.01 or P<0.05) and combined chelation treatment group (P<0.05) (Figure 2).

DFO may have a better protection effect on bone marrow apoptosis
Our data showed that in composite model group, bone marrow had a more significantly TUNEL-positive expression (P<0.01), in accordance with the increased expression of Bax, Caspase-3, Caspase-9, and PARP (P<0.01) and decreased expression of BCL-2 (P<0.01) when compared to normal control group. Iron chelation treatment with DFX, DFO, or DFX + DFO, all exerted a decreased TUNEL-positive rate (P<0.01), while the single treatment with DFO was the most significant one. DFX treatment increased the BCL-2 expression (P<0.05) and downregulated the expression of Caspase-3 and Caspase-9 (P<0.01 or P<0.05); DFO decreased the expression of Caspase-9 and PARP (P<0.01). In addition, combined treatment decreased Bax and Caspase-9 expression (P<0.05), and increased BCL-2 (P<0.05); nevertheless, the antia apoptotic effect (BCL-2 expression) was

![Figure 1](image-url) Comparison of peripheral hemograms among groups on day +14.
Notes: Routine blood tests were performed 14 days after the establishment of model by using manual classification and counting protocols. Data are shown as mean ± SEM (n=8). *P<0.05, **P<0.01 (as compared with Normal); ***P<0.01 (as compared with DFO). Normal: Normal control group, DFX: DFX-treated group, DFO: DFO-treated group, DFX + DFO: DFO and DFX treated group. Model: Composite model group.

Abbreviations: DFO, deferoxamine; DFX, deferasirox; SEM, standard error of the mean; WBC, white blood cells; Hb, hemoglobin; PLT, platelets.
On day 35, indexes combined iron chelation exerted a more obvious effect when compared to DFO or DFX single-treatment group. The most obvious effect was observed in the DFX group when compared to the model group (P<0.01). Furthermore, the iron deposition was more effective at increasing BMP-6 and hepcidin when compared to the model group (P<0.01 or P<0.05). In addition, no differences in SI, SF, BMP-6, and liver hepcidin expression were found between DFX and DFO groups; however, the DFX + DFO group had a significantly lower SI and higher BMP-6 and hepcidin when compared to either DFX or DFO group (P<0.01 or P<0.05), and a lower SF when compared to DFX group (P<0.05); no notable differences were observed when compared to the normal group (P>0.05). DFO treatment could recover the SF when compared to normal group (P<0.05) (Figure 6).

Moreover, Western blot showed that the model group had a significantly lower expression of hepcidin, BMP-6, SMAD4, and TfR2 when compared to the normal group (P<0.01); both DFX and DFO upregulated the level of hepcidin and SMAD4 (P<0.01), and DFO also increased the expression of BMP-6 and TfR2 (P<0.01 or P<0.05) when compared to the model group, but all were lower than the normal group (P<0.01 or P<0.05). DFX and liver hepcidin were obviously decreased (P<0.01) when compared to the normal group. DFX, DFO, and DFX + DFO groups had a significantly lower level of SI and SF, and higher BMP-6 and hepcidin when compared to the model group (P<0.01 or P<0.05). Furthermore, no differences in SI, SF, BMP-6, and liver hepcidin expression were found between DFX and DFO groups; however, the DFX + DFO group had a significantly lower SI and higher BMP-6 and hepcidin when compared to either DFX or DFO group (P<0.01 or P<0.05), and a lower SF when compared to DFX group (P<0.05); no notable differences were observed when compared to the normal group (P>0.05). DOF treatment could recover the SF when compared to normal group (P<0.05) (Figure 7).

Moreover, Western blot showed that the model group had a significantly lower expression of hepcidin, BMP-6, SMAD4, and TfR2 when compared to the normal group (P<0.01); both DFX and DFO upregulated the level of hepcidin and SMAD4 (P<0.01), and DFO also increased the expression of BMP-6 and TfR2 (P<0.01 or P<0.05) when compared to the model group, but all were lower than the normal group (P<0.01 or P<0.05). DFX + DFO treatment increased the expression of hepcidin, BMP-6, and SMAD4 (P<0.01) when compared to model mice, and had a better effect on upregulating BMP-6 and SMAD4 expression compared to DFX single treatment (P<0.05) and more SMAD4 expression compared to DFO group (P<0.05), but hampered the expression of hepcidin and TfR2 compared to either DFO or DFX group (P<0.01 or P<0.05). DFX showed a greater effect in increasing TfR2 level compared to DFO (P<0.05), and DFO appeared more effective in upregulating BMP-6 expression (P<0.05) (Figure 7).
Figure 3 Comparison of apoptosis in bone marrow among different groups.

Notes: (A) The bilateral femurs were extracted from randomly chosen mice from the groups on day +35 following overnight fasting and euthanasia via cervical dislocation. After TUNEL and immunohistochemical staining, slides were observed under a light microscope and photographed under 10×40 resolution, and 3 random areas were selected. (B) The staining intensity and staining rate was evaluated and scored, and their product was used for further statistical analysis. Data are shown as mean ± SEM (n=3), *P<0.05, **P<0.01 (as compared with Normal); †P<0.05, ††P<0.01 (as compared with Model); ‡P<0.05, ‡‡P<0.01 (as compared with DFX + DFO); †††P<0.01 (as compared with DFO).

Normal: normal control group, DFX: DFX-treated group, DFO: DFO-treated group, DFX + DFO: DFO and DFX cotreated group, Model: Composite model group.

Abbreviations: DFX, deferasirox; DFO, deferoxamine; SEM, standard error of the mean.
Figure 4 Comparison of liver NF-κB expression among different groups.

Notes: (A) Liver expression of NF-κB was detected by Western blot, and the results were analyzed using ImageJ 1.46r software. Relative abundance of the proteins was expressed relative to β-actin. (B) Data are shown as mean ± SEM (n=3). **P<0.01 (as compared with Normal); ††P<0.01 (as compared with Model); Normal: Normal control group, DFX: DFX-treated group, DFO: DFO-treated group, DFX + DFO: DFO and DFX cotreated group, Model: Composite model group.

Abbreviations: DFO, deferoxamine; DFX, deferasirox; SEM, standard error of the mean.

Discussion

The impairment of hematopoiesis due to excessive iron overload may be ascribed to the direct effect of increased reactive oxygen species (ROS) mediated by apoptosis of hematopoietic progenitors as well as hematopoietic microenvironment dysfunction.19–21 Increasing numbers of reports have described hematopoietic improvement after the iron chelation treatment in AA and MDS patients complicated...
DFO can reverse the suppression effect of iron overload on erythroid burst-forming unit colonies formation and lineage differentiation by reducing intracellular ROS levels. DFX exerts an effect on inhibiting NF-κB-mediated gene expression, and decreases the level of activated T-cell-produced TNF, which is detrimental to normal hematopoiesis. Yet, it has also been reported that DFX exposure may induce ROS, which hampers the growth and viability of hematopoietic progenitor cell (HPC) from MDS, and in turn decreases CD34 expression in normal HPC. This shift may lead to the activation of POU5F1, SOX2, and SOX17 gene expression, which promote erythroid differentiation. It appears that the mechanisms underlying DFX in hematopoietic recovery are not similar to that of DFO. Thus, it needs to be clarified if there is a synergistic promotion effect of DFX and DFO on AA in vivo.

Our results showed that iron chelation treatment did promote the hematopoietic recovery in iron-overload AA mice, and DFO exerted a more significant effect on the early stage of the disease. With persistent chelation with DFO, the PLT count also improved. The combination management was not superior to single treatments. Furthermore, we detected the levels of apoptosis in bone marrow and found that the DFO single treatment also had a better effect in reducing TUNEL-positive rate than DFX or combined chelation. The effect of DFO and DFX on altering the apoptotic regulatory proteins’ expression was different. The obtained results showed that the DFO was more effective in decreasing the apoptosis promotion proteins (Bax, caspase-3, and PARP), while DFX was more effective in increasing the antiapoptosis protein expression (BCL-2) and inhibiting caspase-9 expression, and the combination treatment was more effective in decreasing the Bax and caspase-9 expression. The Western blot showed that the expression of NF-κB also decreased in all chelation groups; it seems that the DFO treatment exerted a more obvious effect that the DFX and combination
The effect of DFX and DFO iron chelation in AA and iron overload

Figure 7 Comparison of liver hepcidin regulator expression among different groups.

Notes: (A) Liver expression of hepcidin, BMP-6, SMAD4, and Tfr2 were detected by Western blot, and results were analyzed using ImageJ 1.46r software. Relative abundance of the proteins was expressed relative to β-actin. (B) Data are shown as mean ± SEM (n=8); *P<0.05, **P<0.01 (as compared with Normal); †P<0.05, ‡P<0.01 (as compared with Model); ‡P<0.05, §P<0.01 (as compared with DFX + DFO); ‡P<0.05 (as compared with DFO). Normal: Normal control group, DFX: DFX-treated group, DFO: DFO-treated group, DFX + DFO: DFO and DFX cotreated group, Model: Composite model group.

Abbreviations: DFO, deferoxamine; DFX, deferasirox; SEM, standard error of the mean.

After being transferred to the cytoplasm of intestinal epithelial cells by divalent metal transporter 1, which is absorbed from ingested food, iron is stored in SF or transferred into plasma by ferroportin (FPN). Hepcidin is believed to negatively regulate the transportation of iron into the plasma combined with FPN, and induce its phosphorylation, endocytosis, and degradation in cytoplasm. There are many regulatory mechanisms underlying hepcidin expression, but the HJV-BMP-3 signaling pathway plays one of the most important roles. HJV, a coreceptor of the BMP signal, can combine with type 1 BMP receptor and activate BMP. The phosphorylation of SMAD 1/5/8 can also promote the process of combination to SMAD4, thus forming a complex that can enter the cell nucleus and stimulate hepcidin expression. Tfr2, which is highly expressed in liver, can also positively regulate the BMP signaling pathway and upregulate hepcidin gene expression. In our mice model, iron-overload AA was characterized by a heavy iron deposition in liver and bone marrow and by a significantly higher expression of SI and SF and lower expression of hepcidin as well as its positive regulatory factors (BMP-6, SMAD4, and Tfr2). Iron chelation can reverse this, that is, DFX has...
a better effect in upregulation of BMP-6 and TIR2, while the combined treatment has a more significant effect in reducing the SI and SF and increasing the expression of serum BMP-6, liver hepcidin, and SMAD4.

Conclusion
In conclusion, we found that the both DFO and DFX can promote the recovery of hematopoiesis in iron-overload AA; DFO may have a higher efficiency in improving the WBC and PLT count when compared with DFX. The combination treatment with DFO and DFX could accelerate the chelation of iron, but it did not show a further superiority in improving the hemogram recovery. In addition, there is no significant difference on the iron chelation effect between DFO and DFX. In clinical practice, it is possible to optimize the selection of iron chelation drugs based on a patient’s situation and ongoing treatment.

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Author contributions
Dijiong Wu, Baodong Ye, and Yuhong Zhou conceived and designed the study. Dijiong Wu and Xiaowen Wen developed the methodology. Xiaowen Wen and Huijin Hu assisted in the acquisition of data. Dijiong Wu and Xiaowen Wen analyzed and interpreted the data. Dijiong Wu and Baodong Ye wrote, reviewed, and revised the manuscript. All authors contributed toward data analysis, drafting and critically revising the paper, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure
The authors report no conflicts of interest in this work.

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