IJBC 2013;3: 99-105

Short-term Chelating Efficacy of Deferoxamine in Iron Overloaded Rat Hepatocytes

Kalanaky S[1], Farsinejad A[2]*, Fakharzade S[1], Karbasian MA[1], Keshavarz M[1], Mehrvar A[3], Mehrvar N[4], RahbarM[5], Faranoush M[6]

1. Iranian Blood Transfer Organization, Tehran, Iran.
2. Department of physiology and Cardiovascular Research Center, Kerman University of Medical Sciences, Kerman, Iran.
3. Pediatric hematologist oncologist, MAHAK children Hospital, Tehran-Iran.
4. Research department, MAHAK children hospital.
5. Semnan University of Medical Sciences , Semnan, Iran
6. Pediatric hematologist oncologist, Iranian Blood Transfer Organization, Tehran, Iran.

***Corresponding Author:** Farsinejad A, Email: farsinezhad239@yahoo.com*

Submitted: 06-07-2012 , Accepted: 13-10-2012

Abstract

Background: Iron overload is a clinical consequence of repeated blood transfusions and causes significant organ damage, morbidity, and mortality in the absence of proper treatment. The primary targets of Iron chelators used for treating transfusional Iron overload are the prevention of Iron ingress into tissues and its intracellular scavenging. The present study was aimed at elucidating the capacity of clinically important Iron chelator, deferoxamine to gain access to intracellular Iron pools of key Iron accumulating cells (hepatocytes).

Material and methods: The study was conducted as an in vivo investigation. Iron-rich chow fed rats and regular chow fed rats were given deferoxamine and hepatic Iron concentration was measured using atomic absorption spectroscopy.

Results: In Iron-loaded rats, the results showed that deferoxamine did not alter hepatocyte Iron levels compared with the control group but increased urinary excretion.

Conclusion: We conclude that short term deferoxamine treatment is ineffective in Iron removal from rat hepatocytes.

Key words: Deferoxamine, Iron overload, hepatocytes.

Introduction

Iron overload is one of the most important restriction factors in the life expectancy of patients with homozygous β-thalassemia, sickle cell anemia or refractory anemia who are maintained with multiple transfusions [1]. Two cell types are associated with the storage of excess Iron, hepatocytes and reticuloendothelial cells. Clinical findings indicate that Iron storage in reticuloendothelial cells is relatively harmless and that hepatocyte siderosis is responsible for most of the clinical manifestations of Iron overload [2]. Efficacy, site of action in Iron absorption and Iron release in the body, mechanism of action, side effects and prescription of different Iron chelators are considered from many years ago. Deferoxamine (DFO) has low lipid solubility (Kpart of 0.001) and this, together with its molecular weight of more than 657, retards uptake into cells [3]. Countering these effects, however, is the fact that DFO is positively charged, which will favor uptake into cells due to the negative Nernst potential in vertebrate cells [4]. The extent to which these properties limit or facilitate uptake into different cell types may influence the effectiveness of DFO therapeutically [5]. Moreover, the uptake of a chelator into cells is not in itself sufficient for effective chelation of all cellular Iron pools [6]. Renewed interest in DFO within the last few years has been largely the result of the pioneering work of Barry et al., who showed that it is possible to prevent the accumulation of Iron and the progression of hepatic fibrosis in thalassemic patients by prolonged DFO therapy [7,8,9]. Although evidence exists for a rapidly chelatable but small transient labile Iron pool within most cells, storage Iron is not readily available to any chelator even at concentrations above those achievable clinically[3].

Thus, there are significant pools of Iron that are potentially chelatable from thermodynamic considerations but that are not clinically available because of slow kinetic availability [10]. Researchers have described the effect of DFO on a variety of cellular models such as K562 (Human chronic myelogenous leukemia), HCT-116 (human colon carcinoma cells), HepG2 (human hepatoma cells), J774 (mouse macrophage cells), H9C2 (rat cardiomyocyte) and H4-II-E-C3 (rat hepatoma cells) with different approaches [11-16]. They also have studied long-term effects of DFO in animal models. Nevertheless, there are still controversies surrounding short-term efficacy of Iron chelator DFO. The main objective of this study was to investigate the effect of short-term DFO treatment on rat hepatocytes. Our findings have implications for designing chelation regimens to overcome Iron overload.

Materials and methods

Sixteen male Wistar rats of the Hadassah strain weighting 180-220 grams and aged 6-7 weeks were obtained from Pasteur Institute of Iran. These animals were monitored for 2 months. Animals were housed in groups of four animals in cages. Room lighting and temperature were kept controlled (12h light/dark cycle, 22°-25°C). When animals were adjusted to their new environment, they were divided in two random groups: group 1 and 2. The first group was fed an Iron-rich diet and the second group was fed a normal chewable diet. Each group was then divided into two groups which were named A1, A2, A3 and A4 arbitrarily. Both groups of A1 and A3 were injected with DFO and control rats (A2 and A4 groups) were injected with pyrogen-free saline. The structure of division is outlined in figure 1.

In order to specify the type and the dosage of the Iron-rich diet, a pilot study was conducted before the main test began. Due to the rats' refusal to consume from carbonyl Iron-rich diet, it was decided to feed them with normal chewable diet, dried after soaking in ferrous sulfate solution (Khwarizmi Co., Iran).

Iron loading

Eight rats were fed with routine rodent chew for 7 weeks, and other 8 rats fed with 1% Iron enriched rodent chew for 7 weeks, when their faces became darker [17]. After 49 days, all rats were confined in solitary metabolic cages with stainless-steel grid bottoms and their urine was collected. Rats were housed separately in 16 metabolic cages to collect their urinary excretion. Before being transported, all these cages were acid washed to prevent any Iron contamination. Iron-rich diet was maintained throughout the course of the study. This model of dietary Iron overload is commonly used in experimental work and usually results up to 7-fold increase in serum Iron. However, it is known that a 2-fold increase in serum Iron will result in 5-to-8 fold increase in hepatic tissue Iron content, over a period of 12 to 16 weeks.

Determination of urinary Iron Excretion

The group of Iron- rich chew fed rats (A1) and The group of regular chew fed rats (A3) were injected with DFO (50 mg/day IP, 4 days). At the same time the rest of rats (group A2 and A4) were

Table1: Iron levels in rat hepatocyte. Results show Iron overloading in A1 and A2 groups compared with the controls.

A1 Group		A2 Group		A3 Group		A4 Group	
Rat no.	liver Fe content (ppm)	Rat no.	liver Fe content (ppm)	Rat no.	liver Fe content (ppm)	Rat no.	liver Fe content (ppm)
Rat 1	992	Rat 5	872	Rat 9	165	Rat 13	145
Rat 2	806	Rat 6	712	Rat 10	134	Rat 14	128
Rat 3	988	Rat 7	928	Rat 11	164	Rat 15	154
Rat 4	794	Rat 8	887	Rat 12	132	Rat 16	147
Mean	895	Mean	850	Mean	149	Mean	144
SD	110	SD	95	SD	18	SD	11

injected with normal saline (1 cc/day, ip, 4 days). During these 4 days of injections all rats' urine were collected to determine the Iron content using atomic absorption technique.

Determination of hepatocytes Iron content

At the end of 59th day, all animals were anesthetized by intramuscular injection of xylocaine 0.1 mg/kg and ketamin 0.3 mg/ kg. Their hepatic tissue was harvested and kept in formalin, to be prepared for Iron measurement. For determination of Iron content, 2 grams of hepatic tissue was digested in 2 cc of nitric acid (65%) and was heated at 110°C for 1 hour, then at 160°C for 2 hours, and then at 200°C for 3 hours. Digested samples were diluted with distilled water and samples were analyzed by atomic absorption technique.

Statistical analysis

The differences were determined by Mann-Whitney U test. Differences with $P<0.05$ were considered significant.

Results

Following consumption of Iron-rich diet and at the end of the study period, all animals were alive and did not show any abnormal signs (such as growth retardation).

Liver Iron content

The hepatic Iron levels in Iron-loaded rats and controls were measured after 58 days. According to table 1, the hepatocyte Iron level in A1 and A2 groups, who were fed the Iron-rich diet, compared with control animals in A3 and A4 groups was increased significantly (872±98 ppm vs. 146±14 ppm, $p<0.05$). Also there was no significant difference between the hepatic Iron levels in A1 and A3 groups (who were injected with DFO) compared to internal control groups A2 and A4 groups, who were injected with saline (895±110ppm vs. 850±95ppm, $p>0.05$ and 149±18ppm vs.144± 11ppm, $p>0.05$). In other words, DFO did not change the Iron level in either of A1 or A3 group hepatocytes.

Urinary Iron excretion levels

After 49 days, the Iron-rich diet fed rats, were confined in solitary metabolic cages and their urine was collected in acid-washed glassware dishes. During these 4 days, Iron- rich diet was followed. The urine Iron content measurements are shown in table 2. As it can be observed in figure 2, the comparison of the urine Iron levels in DFO- treated rats group A1 with control rats group A2 (saline-treated) revealed a significant increase in urinary Iron excretion in DFO treatment group A1 (28± 8.2 ppm vs. 13.5± 1.7ppm, $p<0.05$). In conclusion, DFO is effective in removing the Iron through urine excretion.

Discussion

Iron is a necessary substance required by the body for its functioning, while excess accumulation

Table2: Increased urine Iron levels in DFO- treated rats compared with control group after 96 hours.

A1		A2	
Rat no.	Urine Fe content (ppm)	Rat no.	Urine Fe content (ppm)
Rat 1	25.2	Rat 5	14.8
Rat 2	25.3	Rat 6	13.4
Rat 3	32.8	Rat 7	14.7
Rat 4	28.5	Rat 8	11.2
Mean	28	Mean	13.5
SD	8.2	SD	1.7

of it causes organ damage through the production of oxygen free radicals. Iron storage and transport is a complex process that is regulated by various factors including hepcidin. As the body does not have a specific mechanism to excrete excessive Iron, it is easily accumulated in those patients requiring multiple transfusions (eg, major thalassemia, sickle cell disease) or in the subjects with congestive failure and an unstable hemodynamic status (eg, hemochromatosis). Iron toxicity can be caused by the labile Iron pool (LIP) within the cells as well as the free Irons, labile plasma Iron (LPI) and non-transferrin-bound Iron (NTBI) in the blood circulation [18]. It is assumed that the most available LIP in cells is associated with the cytosol. The cytosol is also the location where Iron is released from heme and from where Iron is stored into ferritin molecules [19,20]. Treatment of excess Iron storage involves artificial removal of Iron from the body. In this case chelators are administered which bind Iron. The complex of chelator with Iron is excreted in the urine [21]. A commonly used chelator is DFO. Although DFO has been used in the clinic for many years and new generations are in pre-clinical evaluation stages, our knowledge about the modes by which it gain access to cellular Iron pools is limited[14]. Understanding these modes is essential not only for assessing chelator efficacy, but also to produce new drugs with improved biomedical properties. Deferiprone (DFP) and exjade (deferasirox), the new oral chelators were developed as small molecules that have high membrane penetration abilities and thereby also high cell Iron extraction capacity [22]. However, the fact that DFO with the hydrophilic features and relatively large size has none of cell membrane penetration properties, but it is still considered as a safe and effective in removing Iron from the body suggests that other variables in addition to membrane factors might also be involved in Iron chelation. Previous investigations have revealed that DFO is effective in reversing hepatic Iron overload and reducing serum ferritin levels. Long-term administration of DFO is associated with a notable reduction in cardiac complications and increase longevity [14]. In this study, short-term effect of DFO administration in removing Iron from the Iron-loaded rat hepatocytes was investigated. Since the amount of Iron in hepatocytes is a measure of the hepatic Iron burden, atomic absorption spectroscopy was applied to quantify liver Iron. To assess the effect of DFO on LIP, urinary Iron excretion (UIE) were also determined. The results revealed that DFO is unable to remove excessive liver Iron deposition in overload conditions. Comparing the hepatocyte Iron level in Iron overloaded rats who had DFO-treated with other group who had no DFO, showed that DFO is unable to remove Iron deposition from rats' hepatocytes. The present study also revealed that following DFO administration, UIE was significantly higher in the Iron-loaded animals compared with the control group. Results of previous reports on UIE in response to DFO are in agreement with ours [23,24]. Our findings are consistent also with a two-compartment model for effective Iron chelation. The first is a rapid removal of labile cellular (and plasma) Iron which is necessary for reversal of heart failure and is likely to be responsible for the rapid improvement in many cases using DFO [25,26]. The second is the slower removal of storage Iron, either ferritin or hemosiderin, which while not directly harmful to cells, will provide Iron to the labile Iron pool as it is regularly turned over and will thereby contribute to cell damage if a chelator is not present in Iron-loaded cells on a continuous basis. Before us, researchers have examined impacts of DFO on HepG2 (The cell lines of human hepatoma)

Figure 1: Classification of DFO treated rats.

with similar results. Glickstein et al. in an in vitro study indicated that chelation by deferoxamine in HepG2 is slow [14]. However, deferoxamine has different effects on other cells. K562 cells (Human erythromyeloblastoid leukemia cell line) are a convenient in vitro model to study the interaction of chelators with transferrin-delivered Iron. Porter et al. observed that DFO is able to chelate Iron from K562 cells [3]. Our findings are consistent also with the studies indicating that optimal cardioprotection in thalassemic patients is achieved by sequential nightly treatments of subcutaneous DFO [27-30]. Zanninelli et al. showed that steady-state LIP levels in untreated hepatocytes were raised by 1.8-fold following Iron loading and were reduced by 0.66-fold by short-term chelation treatment (The labile Iron pool of) while the Iron storage was intact [31]. These observations overall are consistent with insignificant effect of short term administration of DFO on Iron stores (either ferritin or hemosiderin), although more conclusive results need greater sample size. This study supports the hypothesis that DFO has more access to LIPs than Iron stored in ferritin molecules, hence hypothetically seems that concomitant use of DFO with hepcidin antagonists such as fursultiamine, a Food and Drug Administration (FDA)-approved thiamine derivative, could promote release of Iron deposits and thereby help to removal of excess Iron from the body. This hypothesis should be investigated in the future.

Conclusion

We conclude that short term deferoxamine treatment is ineffective in Iron removal from rat hepatocytes.

References

1. Gary M. Brittenham. Iron-Chelating Therapy for Transfusional Iron Overload. N Engl J Med. 2011; 364(2): 146–156.
2. Hershko C, Konijn AM, Link G. Iron chelators for thalassaemia. Br J Haematol. 1998;101(3):399-406.
3. Porter JB, Rafique R, Srichairatanakool S, Davis BA, Shah FT, Hair T, et al. Recent insights into interactions of deferoxamine with cellular and plasma iron pools: Implications for clinical use. Ann N Y Acad Sci. 2005;1054:155-68.
4. Singh S, Hider RC, Porter JB. Separation and identification of desferrioxamine and its iron chelating metabolites by high-performance liquid chromatography and fast atom bombardment mass spectrometry: choice of complexing agent and application to biological fluids. Anal Biochem. 1990;187(2):212-9.
5. Hershko C, Rachmilewitz EA. Mechanism of desferrioxamine-induced iron excretion in thalassaemia. Br J Haematol. 1979;42(1):125-32.
6. Brady MC, Lilley KS, Treffry A, Harrison PM, Hider RC, Taylor PD. Release of iron from ferritin molecules and their iron-cores by 3-hydroxypyridinone chelators in vitro. Inorg Biochem. 1989;35(1):9-22.
7. Pepe A, Meloni A, Capra M, Cianciulli P, Prossomariti

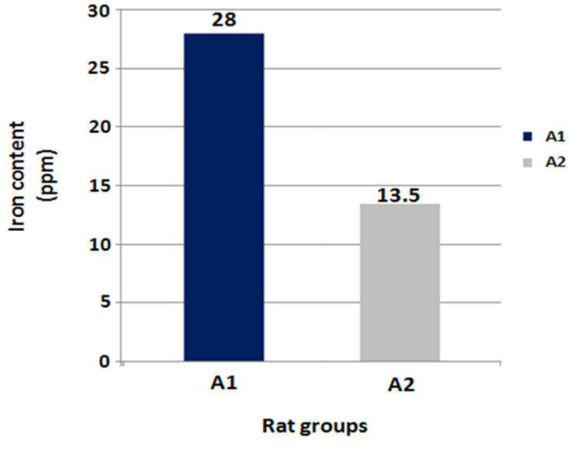

Figure 2: The comparison of the mean urine Iron levels in DFO-treated rats with saline-treated group.

L, Malaventura C, et al. Deferasirox, deferiprone and desferrioxamine treatment in thalassemia major patients: cardiac iron and function comparison determined by quantitative magnetic resonance imaging. Haematologica. 2011;96(1):41-7.
8. Roberts DJ, Rees D, Howard J, Hyde C, Alderson P, Brunskill S. Desferrioxamine mesylate for managing transfusional iron overload in people with transfusion-dependent thalassaemia. Cochrane Database Syst Rev. 2005;(4):CD004450.
9. Caro J, Huybrechts KF, Green TC. Estimates of the effect on hepatic iron of oral deferiprone compared with subcutaneous desferrioxamine for treatment of iron overload in thalassemia major: a systematic review. BMC Blood Disord. 2002;2(1):4.
10. Nisbet-Brown E, Olivieri NF, Giardina PJ, Grady RW, Neufeld EJ, Séchaud R, et al. Effectiveness and safety of ICL670 in iron-loaded patients with thalassaemia: a randomised, double-blind, placebo-controlled, dose-escalation trial. Lancet. 2003;361(9369):1597-602.
11. Wang Z, Zhou HJ. Dihydroartemisinin down-regulates the expression of transferrin receptor in myeloid leukemia cells. Yao Xue Xue Bao. 2008;43(6):576-83. (Abstract)
12. Turner J, Koumenis C, Kute TE, Planalp RP, Brechbiel MW, Beardsley D, et al. Tachpyridine, a metal chelator, induces G2 cell-cycle arrest, activates checkpoint kinases, and sensitizes cells to ionizing radiation. Blood. 2005;106(9):3191-9.
13. Fang C, Zhao C, Liu X, Yang P, Lu H. Protein alteration of HepG2.2.15 cells induced by iron overload. Proteomics. 2012;12(9):1378-90.
14. Glickstein H, El RB, Shvartsman M, Cabantchik ZI. Intracellular labile iron pools as direct targets of iron chelators: a fluorescence study of chelator action in living cells. Blood. 2005 Nov 1;106(9):3242-50.
15. 15. Gao X, Campian JL, Qian M, Sun XF, Eaton JW. Mitochondrial DNA damage in iron overload. J Biol Chem. 2009;284(8):4767-75.
16. Kidane TZ, Sauble E, Linder MC. Release of iron from ferritin requires lysosomal activity. Am J Physiol Cell Physiol. 2006;291(3):C445-55.
17. Panjrath GS, Patel V, Valdiviezo CI, Narula N, Narula J, Jain D. Potentiation of Doxorubicin cardiotoxicity by iron loading in a rodent model. J Am Coll Cardiol. 2007;49(25):2457-64.
18. Kohgo Y, Ikuta K, Ohtake T, Torimoto Y, Kato J. Body iron metabolism and pathophysiology of iron overload. . Int J Hematol. 2008;88(1):7-15.
19. Espósito BP, Epsztejn S, Breuer W, Cabantchik ZI. A review of fluorescence methods for assessing labile iron in cells and biological fluids. Anal Biochem. 2002;304(1):1-18.
20. Kakhlon O, Cabantchik ZI. The labile iron pool: characterization, measurement, and participation in cellular processes(1). Free Radic Biol Med. 2002;33(8):1037-46.
21. Shander A, Cappellini MD, Goodnough LT. Iron overload and toxicity: the hidden risk of multiple blood transfusions. Vox Sang. 2009;97(3):185-97.
22. Neufeld EJ. Oral chelators deferasirox and deferiprone for transfusional iron overload in thalassemia major: new data, new questions. Blood. 2006;107(9):3436-41.
23. Giardina PJ, Grady RW. Chelation therapy in beta-thalassemia: an optimistic update. Semin Hematol. 2001;38(4):360-6.
24. Collins AF, Fassos FF, Stobie S, Lewis N, Shaw D, Fry M, et al. Iron-balance and dose-response studies of the oral iron chelator 1,2-dimethyl-3-hydroxypyrid-4-one (L1) in iron-loaded patients with sickle cell disease. Blood. 1994;83(8):2329-33.
25. Anderson LJ, Westwood MA, Holden S, Davis B, Prescott E, Wonke B, et al. Myocardial iron clearance during reversal of siderotic cardiomyopathy with intravenous desferrioxamine: a prospective study using T2* cardiovascular magnetic resonance. Br J Haematol. 2004;127(3):348-55.
26. Davis BA, Porter JB. Long-term outcome of continuous 24-hour deferoxamine infusion via indwelling intravenous catheters in high-risk beta-thalassemia. Blood. 2000;95(4):1229-36.
27. Origa R, Bina P, Agus A, Crobu G, Defraia E, Dessì C, Leoni G, et al. Combined therapy with deferiprone and desferrioxamine in thalassemia major. Haematologica. 2005;90(10):1309-14.
28. Anderson LJ, Westwood MA, Prescott E, Walker JM, Pennell DJ, Wonke B. Development of thalassaemic iron overload cardiomyopathy despite low liver iron levels and meticulous compliance to desferrioxamine. Acta Haematol. 2006;115(1-2):106-8.
29. Pennell DJ, Berdoukas V, Karagiorga M, Ladis V, Piga A, Aessopos A, et al. Randomized controlled trial of deferiprone or deferoxamine in beta-thalassemia major patients with asymptomatic myocardial siderosis. Blood. 2006;107(9):3738-44.
30. Borgna-Pignatti C, Cappellini MD, De Stefano P, Del Vecchio GC, Forni GL, Gamberini MR, et al.

Cardiac morbidity and mortality in deferoxamine- or deferiprone-treated patients with thalassemia major. Blood. 2006;107(9):3733-7.

31. Zanninelli G, Loréal O, Brissot P, Konijn AM, Slotki IN, Hider RC, et al. The labile iron pool of hepatocytes in chronic and acute iron overload and chelator-induced iron deprivation. J Hepatol. 2002;36(1):39-46.