Forms of Iron Binding in the Cells and the Chemical Features of Chelation Therapy

S. Puntarulo* and M. Galleano

Physical Chemistry-PRALIB, School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina

Abstract: Iron is essential for human life, however it can be toxic through Fe ability to generate oxygen-derived radicals. This work reviews the main features of Fe binding in the cell and its association to these processes. The chemical nature of the Fe extracted by chelation therapy in pathophysiological situations is also analyzed.

1. INTRODUCTION

Fe is an essential element for the growth and well-being of almost all living organisms [1]. Fe is involved in many biological functions, such as DNA synthesis and respiration, due to all the basic processes requiring enzymes with Fe as a cofactor. By spanning the standard redox potential range through varying the ligands to which it is coordinated, Fe participates in oxygen transport, activation and detoxification [2]. As a consequence of its one-electron transfer capacity, in addition to its abundance on earth's crust and the high ferrous salts solubility, Fe was widely utilized by the first stages of life, in the absence of oxygen [3]. When the earth's environment became aerobic majority of ferrous salts were oxidized to ferric salts, that are very insoluble in aqueous solution at pH 7.0 ($K_{spFe}^{3+}= 10^{-17}$ M) [4]. Since then, living organisms had to develop strategies to maintain Fe available for essential processes avoiding or minimizing its toxic effects. Fe toxicity is ascribed essentially to its ability to catalyze the conversion of normal by-products of cell respiration, like superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), into highly damaging hydroxyl radical (•OH) through the Fenton reaction (reaction 1) or by the Fe^{2+} catalyzed Haber-Weiss reaction (reaction 2), or into equally aggressive ferryl ions or oxygen-bridged Fe^{2+}/Fe^{3+} complexes [5]. Fe^{3+} can be reduced either by O_2^- (reaction 3) or by ascorbate or any other reductant agent leading to further radical production.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + \bullet OH \qquad \text{Reaction 1}$$

$$O_2^- + H_2O_2 \xrightarrow{Fe} O_2 + HO^- + \bullet OH \qquad \text{Reaction 2}$$

$$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2 \qquad \text{Reaction 3}$$

Defense against the toxic effect of Fe and O_2 mixtures in humans is provided by two specialized Fe-binding proteins: the extracellular transferrins (Tfs) and the intracellular ferritins (Fts). Both retain Fe in the form of Fe^{3+}, which unless mobilized, will not be able to efficiently catalyze the production of free radicals. Under physiological conditions these proteins would be enough defense, but several situations can produce an increase in total body Fe or a mobilization between compartments, overwhelming the Fe binding capacity. As humans lack a physiological mechanism for eliminating Fe from the body, any increase in the total Fe of the organism is potentially toxic, and pharmacological strategies have been developed to improve Fe chelating therapies.

2. WHICH ARE THE FORMS OF Fe THAT CAN BE CHELATED AND ELIMINATED FROM THE BODY?

In order to identify the possible sources of chelatable Fe, Fig. (**1**) briefly summarizes the main aspects of Fe trafficking and storage in humans. Fe is taken from the diet by duodenal enterocytes as heam and non heam-forms, through independent pathways, and delivered into the bloodstream [6]. Fe incorporation to cells occurs primarily through transferrin-receptors (TfR). TfR are internalized and an endosomal proton pump lowers the pH in the endosome to pH 5-6, and as a consequence the affinity between Tf and Fe is decreased. Thus, the metal is removed from Tf and transported across the endosomal membrane to the cytosol by a divalent metal transporter (DMT1). The TfR bound to the apo-Tf is recycled and apo-Tf is released to bloodstream when the complex returns to the cell surface. Once in the cytosol, Fe is bound rapidly to a variety of compounds to avoid its precipitation due to its low solubility at pH 7 [6]. Little is known about the mechanism of Fe export from the cell but it has been proposed that Fe ions might leave the cells as a complex with low-molecular-weight thiols or/and nitric oxide (NO) [7]. A major fraction of the Fe that entered into the cell would be very fast inserted in Ft, the main protein designed to storage Fe in a safe way. Ft has evolved a molecular design that limits the Fe chemistry within its interior, avoiding non-specific Fe oxidation and hydrolysis reactions from occurring within the cytosol of the cell. In this way other proteins and nucleic acids are protected from the toxic effects of labile Fe. However, in the mammalian cell, Fe stored in Ft can be, and it could be, reactive in initiating lipid peroxidation [8]. This Fe is reduced effectively by reductants with redox potentials more negative than about -200 mV, thus O_2^- (redox potential -300 mV) has the potential to reduce the Fe in the Ft in a hydrofobic environment. Most O_2^- generated by

*Address correspondence to this author at the Fisicoquímica, Facultad de Farmacia y Bioquímica, Junín 956, Buenos Aires, C1113AAD, Argentina; Tel: + 54 11 4964-8244 Ext 101; Fax: + 54 11 4508-3646 Ext 102; E-mail: susanap@ffyb.uba.ar

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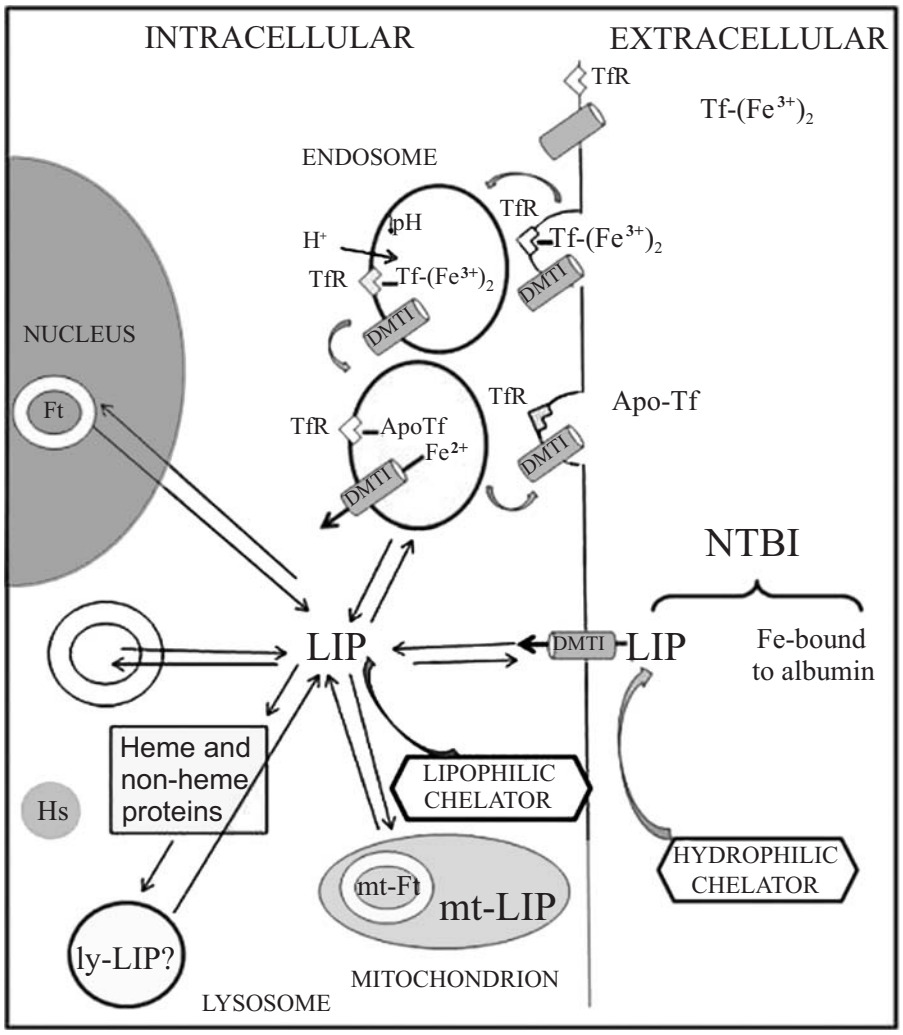

Fig. (1). Fe distribution, trafficking, and storage at extracellular and intracellular levels. Tf $(Fe^{3+})_2$, diferric transferrin; DMT1, divalent metal transporter; Apo-Tf, apotransferrin; Ft, ferritin; Hs, hemosiderin; LIP, labile iron pool; ly-LIP, intralysosomal LIP; mt-Ft, mitochondrial Ft; and mt-LIP, mitochondrial LIP.

microsomal systems arises from decay of the oxygenated P_{450} complex, and as a consequence it would seem likely that P_{450} complex is a major microsomal enzyme responsible for the release of Fe from Ft [9]. Data on microsomal chemiluminescence (CL) and 2,7-dichlorofluorescein diacetate (DCFDA) oxidation by microsomes, shown in Table **1**, suggest a role for microsomal cytochrome P_{450} on the release of catalytic active Fe from Ft. The induction of cytochrome P_{450} isozymes by treatment *in vivo* with either 4-methyl pyrazole (MP; CYP2E1 and CYP2B1/B2 inducer) or phenobarbital (PB; CYP2B1/B2 inducer) increased Ft-dependent radical generation (Table **1**). The increase in DCFDA oxidation by Ft was completely inhibited by anti CYP2B1/B2 IgG, and partially inhibited by anti CYP b_5 IgG suggesting that in the microsomes from PB treated animals the stimulation of the dye oxidation by Ft is mostly due to the activity of CYP2B1/B2 by providing $O_2^{\cdot-}$. On the other hand, Ft could also stimulate the inactivation of enzymes, such as microsomal glucose-6-phosphatase, by a reaction which appeared to reflect the Ft stimulated lipid peroxidation resulting in cellular damage [10]. Moreover, NO chelates labile Fe in a form which decreases its potential to yield reactive intermediates [8]. In this scenario, the prevention of the Ft-dependent stimulation of oxidative reactions by NO [11] suggests that the antioxidant capacity of NO could also involve its ability of decreasing the activity of Fe-heme compounds, such as cytochrome P_{450}, preventing the release of catalytic active Fe from storage forms such as Ft, and thus decreasing the cellular ability to generate free radicals involved in cytotoxicity.

However, Fe must be available to the various cell compartments to fulfil its functions, so Fe must be efficiently released from storage proteins. This Fe is bound to low-molecular-weight molecules giving a steady state concentration of labile Fe within the cell [12]. Labile Fe pool (LIP) is defined as a low-molecular-weight pool of weakly chelated Fe that rapidly passes through the cell. It likely consists of both forms of ionic Fe (Fe^{2+} and Fe^{3+}) associated with a variety of ligands with low affinity for Fe ions. LIP represents only a minor fraction of the total cellular Fe (3-5%) [7]. This intracellular LIP has been extensively studied and it has been

Table 1. Effect of Ft Supplementation on Microsomal Oxidative Reactions

Assays	Microsomes	Ferritin Effect (% Over Control)	Ref.
CL	Saline	52	[9]
	MP	90	[11]
	PB	81	[11]
DCFDA oxidation	Saline	27	[11]
	MP	29	[11]
	PB	56	[11]
+ AntiCYP 2B1/B2 IgG	PB	0	[11]
+ AntiCYP b5 IgG	PB	20	[11]

Measurements were performed on reaction systems including saline microsomes in the presence of 2 μg Ft/mg microsomal prot.

CL stands for chemiluminescence; DCFDA stands for 2,7-dichlorofluorescein diacetate; MP microsomes stands for microsomes prepared from 4-methyl pyrazole treated animals and PB microsomes stands for microsomes prepared from phenobarbital treated animals.

demonstrated that this pool is associated with physiological, pharmacological and toxicological Fe functions. LIP supplies Fe for the synthesis of Fe-containing enzymes, for the functioning of the cellular Fe transport and storage, and for signaling the expression or repression of Fe-responsive genes [13]. However, the chemical nature of the LIP has not been defined yet. It has been proposed that Fe is complexed by diverse low-molecular-weight chelators, such as citrate and other organic ions, phosphate, carbohydrates and carboxylates, nucleotides and nucleosides, polypeptides and phospholipids [14-16]. Among these intracellular chelators a distinctive role for ATP should be pointed out. Besides its central feature as a key factor in the energetic metabolism, this chelate is a good catalyst (when forming an Fe-complex) for lipid peroxidation and microsomal chemiluminescence [17]. Under these circumstances, Woodmansee and Imlay (2002) [13] proposed to define the pool simply as the intracellular Fe that is not stably integrated into enzymes and/or Fe-storage and transport proteins. This definition would be in agreement with the chemical basis of the Fe-complexes reactivity in terms of Haber-Weiss reaction. Only the Fe that has, at least, one coordination site open or occupied by a readily dissociable ligand would be able to participate in this reaction. Fe tightly bound to transport and storage proteins shows all its six coordination sites occupied, therefore it could not act as an active element in catalysis.

The situation is different at the extracellular (vascular) compartment, where Tf is the principal specific Fe-binding protein. Normal adult human plasma contains about 3 to 4 mg of Fe, essentially all bound to Tf [18]. Under normal conditions, serum Tf is 20 to 35% saturated with Fe. However, the Fe-binding capacity of Tf may be exceeded, leading to the appearance of non Tf-bound Fe (NTBI) [19]. There is evidence that suggest that DMT1, a transporter with a broad substrate specifity [20], may be a putative NTBI transporter in hepatocytes [21]. Increased plasma NTBI has been found in patients with thalassemia [22] and genetic hemochromatosis [23], in association with the appearance of oxidation products and decrease in plasma antioxidant capacity. The chelates responsible for complexing Fe in NTBI, as well as in the LIP case, are not well defined, but it would involve both non-protein and protein-bound forms. The non-protein ligands appear to correspond to low-molecular-weight organic compounds (ascorbate, phosphate, carbonate, organic acids, amino acids), as was defined for the LIP, but most of the NTBI in plasma is bound to albumin [24]. Le Lan *et al*. (2005) [25] have discriminate between NTBI and LIP as corresponding to different Fe biochemical speciation's, being NTBI in submicromolar order and LIP even lower.

Due to the strong control to avoid the catalytic effect of Fe, this metal is efficiently stored and is not easily either accessed or eliminated. LIP and NTBI would be the primary Fe sources available for chelation. But Fe steady state concentrations would constitute the resultant of multiple dynamic equilibrium, as shown in Fig. (**1**). Chelation and ulterior elimination of Fe-chelate would produce the progressive displacement of Fe from its storage sites, involving at long term, all of the potentially removable metal. During years, the existence of the LPI and the NTBI were only postulated, without any possibility of reliable assessment.

Nowadays, several techniques have been developed and successfully applied in detecting both forms of Fe in different experimental models and patients [25]. At tissular level, electronic paramagnetic resonance (EPR) constitutes a useful tool to detect Fe due to the paramagnetic nature of this metal. Kozlov *et al*. (1992) [26] have developed an assay to determine the amount of "free Fe" (in terms of Fe not tightly bound to enzymes and transport and/or storage proteins) in experimental models. This methodology was successfully applied to study Fe overload effects in rats. The animals were injected with desferrioxamine (DFO) 20 min previous to the sacrifice and liver samples were immediately frozen at 77K [27]. DFO rapidly chelates Fe^{3+} with high affinity, being able to change the heterogeneous condition of the Fe^{3+} present in the LIP into a unique complex DFO-Fe^{3+}. Additionally DFO favors oxidation of Fe^{2+} and, obviously, will chelate the product. DFO-Fe^{3+} can be detected by EPR at 77K (Fig. (**2**)) and represents the total amount of Fe^{3+} and Fe^{2+} that can be chelated by DFO under the experimental conditions established. Fig. (**2**) shows the increase in the

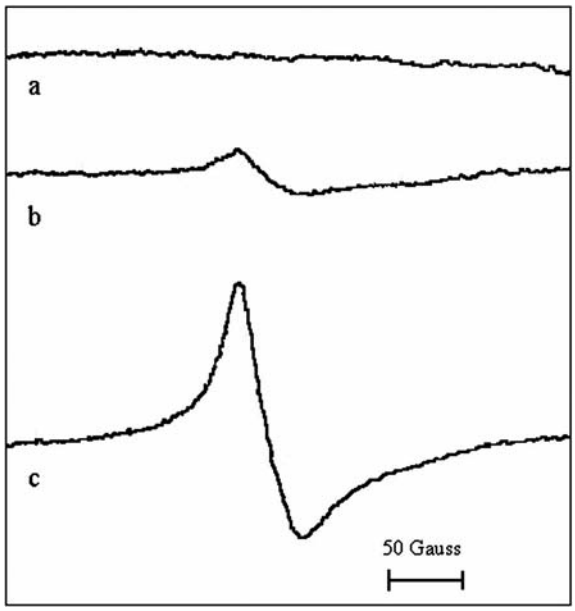

Fig. (2). DFO-Fe^{3+} spectrum (g = 4.3) was measured in liver from control rats without DFO (a); control rats injected with DFO (0.5 mg/kg, ip) 20 min previous to sacrifice (b); and Fe overloaded rats (6 h post ip administration of 200 mg/kg Fe-dextran) injected with DFO (0.5 mg/kg, ip) 20 min previous to sacrifice (c). Liver samples were introduced in 1 ml plastic syringe and immediately frozen at 77K. Afterwards, the samples were transferred to a Dewar insert and measured by EPR. EPR measurements were carried out in a Bruker (Karlsruhe, Germany) espectrometer ECS 106 with a ER 4102ST cavity. Spectrometer settings were: field modulation frequency, 50 kHz; microwave frequency, 9.42; modulation amplitude 4.75 gauss; microwave power 20 mW; time constant, 164 ms; center field 1600 gauss, and sweep width, 800 gauss.

EPR spectrum assigned to DFO-Fe^{3+} in the liver of control rats (trace b) as compared to that recorded in Fe overloaded rats (6 h post ip injection of 200 mg/kg Fe-dextran) (trace c). The quantification of the obtained EPR spectra showed non detectable values in the absence of DFO administration. The amount of labile Fe was 21±5 nmol/g of liver for control animals injected with DFO and 180±30 nmol/g of liver for Fe-overloaded animals injected with DFO (Fig. (**2**)). Thus, this methodology is capable of reflecting the expansion of the Fe pool available to be chelated in a Fe excess condition, besides a possible overestimation of the individual values.

The accessibility of cellular Fe to chelators (such as DFO) is commonly used as the criterion of "lability". In cell lysates, loosely bound Fe representing LIP may be scavenged by weaker or non-penetrant ligands including nitrile triacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA), ATP, Tris and glycine. Petrak and Vyoral (2001) [28] have shown that the choice of the Fe chelator changes the detected amount of Fe in the LIP. The affinity of a chelator for Fe, its ability to permeate different cell compartments as well as the steric accessibility of specific metal binding sites on proteins are the factors that could influence the assessment of labile pool. Regarding intracellular distribution, it has been reported substantial amounts of LIP measured in mitochondria (5-9 μM), nucleus (7-12 μM) and lysosomes (16 μM) [29]. Recently, it has been proposed a relevant role for the lysosomal LIP and its translocation to mitochondria seems to be responsible for oxidative stress-induced hepatocellular injury [30].

Ft plays a dual role in LIP homeostasis. In Fe rich conditions Ft acts as a Fe sequestering protein, protecting cells against Fe toxicity and at low Fe conditions it acts as a source of Fe ions required for the synthesis of Fe containing proteins. Fe is stored mainly intracellularly, where its potentially damaging effects are greatest. Thus, Fts play a key role in preventing Fe toxicity because of their ability to sequester several thousand Fe atoms in their central cavity in a soluble, non-toxic bioavailable form. However, there are indications that Fts may have other functions, not described yet, in addition to the well assessed role in storing intracellular Fe. Ft molecule is a hollow protein shell (outside diameter 12-13 nm, inside 7-8 nm, MW about 500000), composed of 24 polypeptide chains and capable of storing up to 4500 Fe^{3+} atoms of inorganic complex [31]. In mammalian Ft there are two different polypeptide chains known as H and L, and about 90% of H-chain residues and about 85% of L-chains are identical [1]. Ft isolated from mammalian tissues consists of a mixture of isoFts with a range of subunit compositions and Fe contents. In general, L-rich Fts are characteristics of organs storing Fe (liver and spleen) and these Fts usually have a relatively high average of Fe content (1500 Fe atoms/molecule or more), meanwhile, H-rich Fts which are characteristics of heart and brain have relatively low average of Fe content (less than 1000 atoms/molecule) [1]. Such a high Fe:protein ratio (200 times that in hemoglobin) is made possible by sequestering Fe as a compact mineral. The fact that mineralization occurs within preformed intact shells, which limit the size of the hydrous ferric oxide particles, may also influence the availability of Fe. Because of the large surface to volume ratio of the mineral core, both the surface free energy and the free energy of the bulk mineral phase contribute to the stability of the nanoparticle phase of Ft [32].

3. CLINICAL CONDITIONS REQUIRING Fe CHELATION THERAPY

Hereditary hemochromatosis (HH) is a very common genetic defect in the Caucasian population, with an autosomal recessive inheritance. It is characterized by inappropriately increased Fe absorption from the duodena and upper intestine, with consequent deposition in various parenchymal organs, notably the liver, pancreas, heart, pituitary gland and skin [33]. The progression rate of Fe overload in HH individuals in the general population is unknown [34]. Fe overload is characterized by the presence of several clinical manifestations such as: increased susceptibility to infections, hepatic dysfunction, tumors, joint diseases, myocardiopathy, and endocrine alterations. Chelation therapy has long been regarded as a suitable approach to the therapeutic and even prophylactic removal of potentially toxic Fe [35]. In conditions of Fe overload in which phlebotomy can be used, the procedure may provide normal life expectancy. Fe overload has been also observed (a) if dietary Fe is excessive, such as in the severe Bantu siderosis, reported in the Bantu tribe of Africa who drink acidic beer out of Fe pots, (b) in other inherited diseases, such as congenital atransferrinemia (lacking

circulating Tf), and (c) during the medical treatment of thalassemia. Thalassemias are genetic disorders in which the rate of synthesis of one or more of the hemoglobin chains is diminished. Untreated patients die of anemia in infancy but can be kept alive by regular blood transfusions. Since each unit of blood contains about 0.2 g of Fe, the patients become overloaded with Fe and the plasma LIP/NTBI is drastically increased. This is consistent with the following observations concerning the Fe-overloaded plasmas: LIP/NTBI is detectable by the bleomycin assay, •OH production is promoted, peroxidation of liposome phospholipids is stimulated, Tf is completely saturated, and addition of ApoTf is completely protective [36]. It has been recently reported that plasma LIP/NTBI can serve as an early indicator of Fe overload in beta-thalassemia/HbE patients [37].

It was proposed that enhanced synthesis of Ft or removal of Fe from Ft could improve the current regimens for Fe chelation therapy in cases of transfusional Fe overload associated with genetic anemias [38].

Increased serum Ft levels have been associated with the severity of stroke [39] and of coal workers' pneumoconiosis [40], also with acute renal failure [41], and the development of subacute thyroiditis [42] among other pathological situations. Berberat *et al*. (2003) [43] identified anti-apoptotic functions of H-Ft and suggested that H-Ft can be used in a therapeutic manner to prevent liver damage and thus, maximize the organ donor pool used for transplantation. Table **2** summarized several clinical conditions currently associated to Fe overload. Moreover, clinical and epidemiologic observations indicated that increased Fe storage status is a risk

Table 2. Clinical Conditions Associated to Fe Overload

Pathological Condition	Main Features	Ref.
Non-hereditary		
Infection	Fe overload enhances microbial infection, interferes with chemotaxis, phagocytosis, and the microbicidal action of leukocytes, reduces the migration of B and T lymphocytes into the lymphatic system, the number of interleukin-2 secreting cells and T-helper cells, the activity of natural killer cells and the tumoricidal action of macrophages and leads to advanced hepatic fibrosis in patients with chronic hepatitis C.	[66,67]
Neoplasia	Tumor sites could be associated with the deposition of Fe: sarcomas at sites of intramuscular injection of Fe, primary hepatocellular carcinoma in hemochromatotic, siderotic, and/or alcoholic patients, respiratory tract neoplasia and colorectal cancer.	[66-70]
Cardiomyopathy	Chronically stressed adults with excess Fe mostly died by heart failure.	[66,71]
Chronic diseases	Fe is accumulated in patients with chronic liver diseases, obesity, type II diabetes and hypertension.	[72-75]
Hereditary		
Primary hemochromatosis	Is caused by a mutation of aminoacid 282 (C282Y) in the HFE gene. HFE is a protein that appears to be necessary for normal Fe deposition in reticuloendothelial cells. Is characterized by an increase in dietary Fe absorption with a chronic rise in body Fe stores.	[18]
Thalassemias	These disorders are characterized by different genetic alterations that affect the DNA sequence of the human α–like and β–globin genes. Hemoglobin is affected and patients present Fe overload in reticuloendothelial cells with anemia, requiring transfusions that result in excess Fe in the body.	[76]
Neuroferritinopathy	A dominantly inherited basal ganglia disease that is associated with Fe accumulation in various parts of the brain. The mutation disrupts the C-terminus of the L-chain Ft, affecting its stability and function.	[77]
Friedrich´s ataxia	An autosomal recessive neurodegenerative disorder with a defect in the nuclear gene that encodes frataxin. Patients present Fe accumulation in mitochondria and its subsequent dysfunction.	[78]
Hallerverdon Spatz syndrome (Pantothenate kinase –PANK-defect)	An autosomal recessive neurodegenerative disorder caused by a defect in the gene that encodes PANK that appears to be specifically expressed in the brain. This enzyme is essential in coenzyme A biosynthesis and its lack produces an increase in cysteine that would chelate Fe in a form able to increase the free radical production.	[79-81]
Aceruplasminemia	An autosomal recessive disorder of Fe caused by mutations in ceruloplasmin gene. Patients show parenchymal Fe overload and progressive neurological disease by the effect over Fe trafficking.	[46]

factor in several diseases such as porphyria cutanea tarda and sudden infant death syndrome, among others. However, Armaganijan and Batloumi (2003) [44] reported that serum levels of Ft and of organic Fe indicators were neither risk factors nor risk markers of coronary atherosclerosis.

In the last decade, important advances have been made in the knowledge of conditions that involves localized Fe-overload. Those conditions would include short term processes, as organ or tissue ischemia-reperfusion and local inflammation, as well as progressive pathologies essentially affecting the central nervous system. In the first case, the decompartamentalization of Fe would lead to the expansion of the LIP and the increase of the oxidative damage, being a potential situation where Fe chelators would protect the tissues. In the second case, it has been described an increase in Fe levels in the substantia nigra of Parkinsonian brains [45], Hallervorden-Spatz syndrome [46] and in mitochondria of Friedrich´s ataxia cerebella [47]. Chelation therapy of Fe from those particular compartments constitutes a wide field under active research.

4. SEARCHING FOR A BETTER CHELATING AGENT

In designing Fe chelators for clinical application two different aspects should be considered: i) chelating chemical aspects, including metal selectivity, complex stability and redox activity, and ii) pharmacological aspects, such as administration routes, ability of the compound to reach the target site and toxicity. An important number of chemical families have been studied in order to find molecules able to fulfil the requirements for optimal performance as Fe chelators *in vivo*: hydroxamic acids, aminocarboxylates, catechols, hydroxypyridones, pyridoxal isonicotinoyl hydrazone, desferrithiocins, triazoles, dialkylhydroxypyridinones, and hydroxypyridinone derivatives, extensively reviewed by Hershko and Weatherall (1988) [48]; Liu and Hider (2002) [49], and more recently by Crisponi and Remelli (2008) [50].

Despite the efforts, to date, DFO (Desferal®), deferiprone (DFP) or L1 (Ferriprox®) and deferasirox (DFS) or ICL670 (Exjade®) are the only products currently used in therapeutics [51]. DFO is a tris-hydroxamic acid derivative (Fig. (**3**)) that was introduced in the 1970s and constitutes the most widely Fe chelator used in therapy. DFO shows a poor intestinal absorption and a short half-life in plasma (5-10 min) [52]. The current treatment is the continuous parenteral infusion during 8-12 h for 5-7 days at week [53] in a dose 40-50 mg/kg/day. Considering that this therapy requires a high compromise from the patients, two approaches are being explored: (i) new techniques of DFO administration, and, ii) orally active Fe chelators. DFO administration routes tested include: twice daily subcutaneous bolus injections [54], hydroxyethyl starch DFO [55] and DFO depot [56]. Even when, at least the twice daily subcutaneous bolus injection regimen has shown good results, finding of an orally active Fe chelator is the main objective in this field.

DFP and DFS are the orally active Fe chelators currently available for clinical use. DFP is a dialkylhidroxypyridinone (Fig. (**3A**)) that was licensed in India in 1995 and nowadays is available in more than 50 countries [50]. It has been effective administered orally 2 or 3 times per day in a dose of 50-100 mg/kg/day [57]. This high dose is necessary due to the partial transformation into its *O*-glucuronide, eliminating the 3-hydroxyl group, essential for the chelating activity. DFS is a triazol (Fig. (**3**)), approved in 2005, that is administered once a day in a dose of 20 mg/kg/day [58].

The most important feature in the design of a therapeutically useful Fe chelator is the affinity for Fe ions, which is usually defined by their affinity constant (K). However, Fe ions are present in biological systems in two different oxidation states (as divalent and trivalent cations), and this fact established a wide number of cations able to compete with Fe^{2+} and Fe^{3+} for the chelator. Effectiveness of a Fe chelator in living systems face the challenge of trapping Fe from several sources (LIP, storage compartments) without removing it from biologically relevant enzymes and, additionally must be able to have a differential affinity between Fe^{2+} and other divalent cations essential for life [49]. Considering that trivalent cations (e.g. Al^{3+}, Ga^{3+}, In^{3+}) are not physiologically indispensable, and divalent cations (e.g. Cu^{2+}, Zn^{2+}, Mg^{2+}, Co^{2+}) have important functions, selectivity is essential to avoid toxicity by depletion of particular metals [50]. DFO, DFP and DFS have high affinity for Fe, particularly by the trivalent form (Table **3**), while compounds like diethylenetriaminepentaacetic acid (DTPA) or N,N-dimethyl-2-3-dihydroxybenzamide (DMB) (Fig. (**3B**)) that present similar or higher affinity for Fe^{3+} than DFO (K= 28.0 and 40.2, for DTPA and DMB respectively) showed also a high affinity for Fe^{2+} (K>16) and retain an important affinity for other divalent cations (K>21 for Cu^{2+} and K>13 for Zn^{2+}) [49]. As a consequence, even when theoretically a Fe chelator could be designed indistinctly to bind Fe^{2+} or Fe^{3+}, strong Fe^{3+} chelators have advantages when are applied to biological systems. In fact, DTPA has been used in patients when DFO treatment lead to undesirable side-effects, but due to the lack of selectivity, supplementation with Zn was necessary to avoid toxicity by Zn depletion [59].

Another important feature is the stability of the Fe-complex that is highly related with the number of chelate rings formed in the ligand-metal complex. The maximum stability of a complex is reached when the six Fe coordination positions are present in a single molecular structure, creating a hexadentate ligand, as in the case of DFO, while DFP and DFS are bidentate and tridentate ligands, respectively. However, none of these individual parameters are able to describe entirely the efficacy of a Fe chelator. The parameter pFe^{3+} is introduced to take into account more than one characteristic of the ligands simultaneously, such as the effects of ligand protonation, denticity and metal-ligand stoichiometry. This parameter is the negative logarithm of the concentration of "free Fe^{3+}" that remains in solution in the presence of a total ligand concentration of 10^{-5} M and Fe^{3+} concentration of 10^{-6} M at pH 7.4 [49]. Generally, a $pFe^{3+} \geq 20$ is required for efficient Fe scavenging from biological matrices [50]. DFO and DFS have higher and DFP has lower pFe^{3+} values, as compared with the mentioned limit. However, in spite of being a global indicator, is insufficient to describe completely the behavior of the chelator in biological systems, since the pFe^{3+} values, calculated at 25°C and ionic strength 0.1 M, did not reflect the real ionization

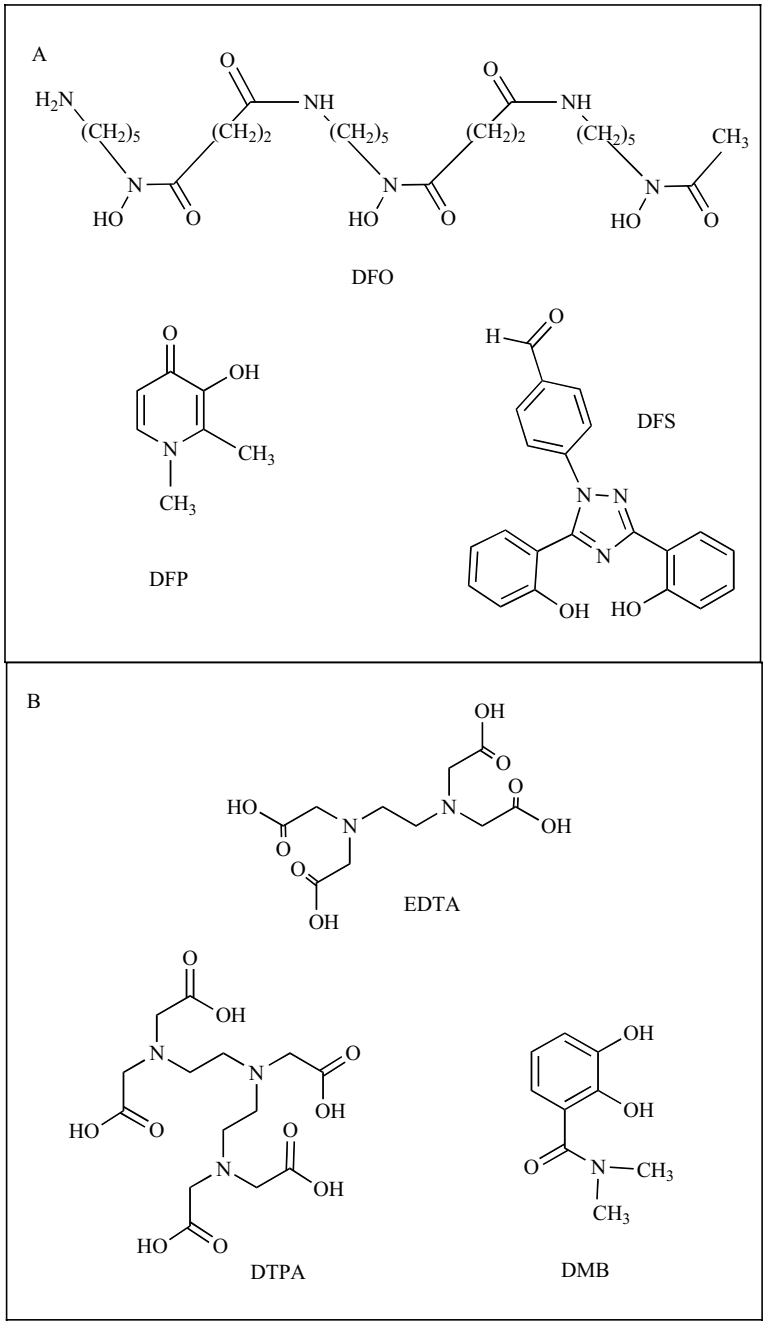

Fig. (3). A. Chemical structures of the Fe-chelating agents currently under clinical use. **B**. Chemical structures of other Fe-chelating agents.

constant under the biological conditions (37°C and 0.15 M ionic strength, respectively) [49].

Other features to be considered to evaluate the toxicity mediated by free radicals production are the redox properties of the final Fe complexes obtained after chelation therapy. Drugs for clinical use should be selected between ligands that form complexes without ability to enter in a redox cycling, such as the following reactions (4 and 5).

$$\text{Ligand-Fe}^{2+} + H_2O_2 \rightarrow \text{Ligand-Fe}^{3+} + {}^{-}OH + \bullet OH \quad \text{Reaction 4}$$

$$\text{Ligand-Fe}^{3+} + AH \rightarrow \text{Ligand-Fe}^{2+} + A + H^{+} \quad \text{Reaction 5}$$

(AH stands for relevant reductant agents)

The ability of a chelator to generate a Fe-complex active or inactive in terms of free radical production is reflected by the reduction potential values ($E^{o\prime}$ in biological systems). This parameter is used to predict the spontaneity of an electron transfer process by calculating the differences between the $E^{o\prime}$ values of the oxidation and reduction half-reactions. A positive $\Delta E^{o\prime}$ indicates an energetically favored reaction, and then there is a range of $E^{o\prime}$ that potentially maintain the Fe redox cycling capacity. For instance, when Fe is complexed with EDTA, it should be potentially able to react with H_2O_2 to produce •OH as indicated by reaction 6.

Table 3. Physicochemical Parameters of the Fe-Chelating Agents Currently Under Clinical Use

Chelator	Metal Affinity		pFe^{3+}	E° (mV)	MW	D
	Fe^{3+}	Fe^{2+}				
DFO	30.6[a]	7.2[a]	26[a]	-450[c]	561	0.01[a]
DFP	37.2[a]	12.1[a]	19[a]	-828[g]	139	0.11[f]
DFS	22.0[e]	Remarkable poor[e]	22.5[d]	-600[b]	373	10[d]

pFe^{3+} corresponds to Fe^{3+}= - log $[Fe^{3+}]$ in solution (where $[Fe^{3+}]_{total} = 10^{-6}$ M and $[ligand]_{total} = 10^{-5}$ M at pH 7.4), E° stands for standard reduction potential, MW stands for molecular weight, and D stands for distribution coefficient.

[a] Liu and Hider, 2002 [49]; [b] Merkofer *et al*., 2004 [82]; [c] Cooper *et al*., 1978 [83], corresponds to E°′; [d] Nick *et al*., 2000 [84]; [e] Steinhauser *et al*., 2004 [85]; [f] Galanello *et al.*, 2007 [57]; [g] El-Jammal and Templeton, 1996 [86].

Thus, Fe^{2+}-complexes with E°′ lower than 320 mV, would render a positive ΔE°′ and will be able to produce •OH. However, to be able to participate in redox cycling, the Fe^{3+}-complex resulting from reaction 6 should be susceptible to reduction by physiologically relevant reductants, such as NAD(P)H, as shown in reaction 7.

	E°′ (mV)	Ref.
$H_2O_2 + e^- \rightarrow {}^-OH + \bullet OH$	320	[60]
$EDTA\text{-}Fe^{2+} \rightarrow EDTA\text{-}Fe^{3+} + e^-$	120	[60]

$EDTA\text{-}Fe^{2+} + H_2O_2 \rightarrow EDTA\text{-}Fe^{3+} + {}^-OH + \bullet OH$ Reaction 6

$\Delta E^{o\prime}$=320–120=+200 mV

	E°′ (mV)	Ref.
$EDTA\text{-}Fe^{3+} + e^- \rightarrow EDTA\text{-}Fe^{2+}$	120	[60]
$\frac{1}{2}$ NAD(P)H $\rightarrow \frac{1}{2}$ $NAD(P)^+ + e^- + \frac{1}{2} H^+$	- 324	[3]

$EDTA\text{-}Fe^{3+} + \frac{1}{2}NAD(P)H \rightarrow EDTA\text{-}Fe^{2+} + \frac{1}{2} NAD(P)^+ + \frac{1}{2} H^+$ Reaction 7

$\Delta E^{o\prime}$=120-(-324)=+444 mV

To be effective as a Fe chelator useful to treat Fe overload disorders, ligands should be able to form safe Fe-chelates, with no capacity to catalyze redox cycling (Note)[1]. The drugs currently under clinical use have reduction potentials lower than -450 mV (Table **3**). Thus EDTA, that is a molecule too small to fully mask the Fe, presents toxic effects [61, 62] as previously described, since effectively catalyzed •OH generation by rat liver microsomes in studies *in vitro* [17].

The effect of Fe chelators on the oxidative stress parameters of Fe overloaded patients has been widely documented. In a recent report, DFO and DFS were comparatively studied in beta-thalassemic patients and oxidative stress was evaluated [58]. Along the twelve month of treatment, plasma malondialdehyde, as an index of lipid peroxidation, declined significantly after the administration of either of these drugs, indicating a similar protection against the free radical damaging effects.

The administration route, a major factor related with the patient comfort and compliance of the treatment, is determined by the ability of the compound to be efficiently absorbed from the gastrointestinal tract and to cross biological membranes in order to reach the targets. Those abilities are related with the molecular weight (MW), the lipophilicity and the net charge. Drugs with MW > 500 present poor absorption, according to the Lipinski criteria [63]. Hider (1995) [64] adjusted that value to MW > 300 taking into consideration the high absorption levels required to a good chelator action. According to this assumption, DFO would require parenteral administration, while DFP and DFS have a good oral absorption (Table **3**).

Lipophilicity can be estimated by the distribution coefficient (D) for ionisable drugs. This parameter, together with the molecular size, conditions both the gastrointestinal ab-

[1] This thermodynamic approach has been made based on the standard condition, which is rarely fulfiled within the cells. A more precise analysis will require the actual concentration of any involved species in the biological system under study and the application of the Nerst equation to obtain the particular E°' for each reaction.

sorption, as the efficient liver extraction and the toxicity. DFO is a highly hydrophilic molecule, with a D = 0.01, and is a good option in terms of toxicity, but its gastrointestinal absorption is very poor, as its liver penetration.

On the other hand, since small chaotropic molecules also open Ft pores in the native structure without altering the global properties of the protein [65], it should be possible to develop new chelators targeted to Ft pores that will remove Fe more efficiently than current chelators since they are mainly targeted to chelate Fe outside the cells or in the LIP.

6. CONCLUSIONS

The following aspects should be considered as the main features of Fe binding in biological systems:

1- Fe is both, an essential element and a toxic compound that has to be carefully kept under a regulated concentration in a living cell.

2- Toxic Fe activity is due to its ability of catalyzing free radical reactions, but not all the Fe forms are equally efficient. The most efficient Fe fraction to act as a free radical promoter is that forming the LIP. LIP concentration is the resultant of multiple dynamic equilibrium between the Fe incorporated to the cell, utilized and intracellularly stored. Therapeutic strategies are designed to chelate either Fe from the LIP or Fe loosely bound to Ft to avoid Fe-related oxidative damage.

3- The main features of the optimal Fe chelator include chemical, as well as pharmacological aspects. Chemical aspects to be considered include Fe-affinity, Fe-selectivity, molecular weight, and lipophilicity, in addition to stability and redox properties of the resultant Fe-complex. Among the pharmacological issues, administration routes, ability of the compound to reach the target site and toxicity are required to be taken into account.

4- Currently clinical research is abording the challenge of designing a Fe chelator with the efficiency of DFO but that could be administered orally to meet critical goals such as good performance and comfort for the patient. Focus in chemical-related aspects of the Fe-chelator complexes should help to fulfil the new drugs designing expectances.

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ABBREVIATIONS

ATP = Adenosine-5'-triphosphate

CL = Chemiluminescence

DCFDA = 2,7-dichlorofluorescein diacetate

DFO = Desferrioxamine

DFP = Deferiprone

DMB = N,N-dimethyl-2-3-dihydroxybenzamide

DMT1 = Divalent metal transporter

DTPA = Diethylenetriaminepentaacetic acid

EDTA = Ethylenediaminetetraacetic acid

E^o = Reduction potential values

$E^{o'}$ = Reduction potential values in biological systems

EPR = Electronic paramagnetic resonance

Ft = Ferritin

HH = Hereditary hemochromatosis

LIP = Labile Fe pool

MP = 4-methyl pyrazole

NO = Nitric oxide

NTA = Nitrile triacetic acid

NTBI = Non Tf-bound Fe

PB = Phenobarbital

Tf = Transferrin

TfR = Transferrin-receptors

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