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Magnetic resonance assessment of iron overload by separate measurement of tissue ferritin and hemosiderin iron

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Abstract

With transfusional iron overload, almost all the excess iron is sequestered intracellularly as rapidly mobilizable, dispersed, soluble, *ferritin iron*, and as aggregated, insoluble *hemosiderin iron* for long-term storage. Established magnetic resonance imaging (MRI) indicators of tissue iron (R2, R2*) are principally influenced by hemosiderin iron and change slowly, even with intensive iron chelation. Intracellular ferritin iron is evidently in equilibrium with the low-molecular-weight cytosolic iron pool that can change rapidly with iron chelation. We have developed a new magnetic resonance imaging (MRI) method to separately measure ferritin and hemosiderin iron, based on the non-monoexponential signal decay induced by aggregated iron in multiple-spin-echo sequences. We have initially validated the method in agarose phantoms and in human liver explants and shown the feasibility of its application in patients with thalassemia major. Measurement of tissue ferritin iron is a promising new means to rapidly evaluate the effectiveness of iron-chelating regimens.

Keywords

iron; ferritin; hemosiderin; thalassemia; magnetic susceptibility; magnetic resonance

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

INTRODUCTION

The application of magnetic resonance imaging (MRI) to the study of transfusional iron overload has focused primarily on techniques to determine the total storage (non-heme) iron concentration in tissues and organs.¹⁻³ We describe here the development of methods to measure separately the two principal types of storage iron, the short-term storage form in ferritin and the long-term deposits in hemosiderin, by using their distinctive effects on MRI signal decay. The unique physiological roles of intracellular ferritin iron⁴⁻⁶ suggest that measurements of this pool within tissues could provide early warning of iron-induced toxicity, permit rapid monitoring of the effectiveness of iron-chelating regimens, and, together with measurements of hemosiderin iron, improve the accuracy of estimates of total storage iron.

STORAGE FORMS OF IRON – FERRITIN AND HEMOSIDERIN

Normally, iron entry into cells is meticulously regulated by controlling uptake of the iron-transport protein transferrin from the plasma via the transferrin receptor on the cell membrane surface.⁷ In patients with thalassemia major and transfusional iron overload, plasma transferrin may become fully saturated with iron and non-transferrin-bound iron (NTBI) may then appear in plasma. This plasma non-transferrin-bound iron can bypass physiological controls and enter vulnerable cells (especially hepatocytes, cardiac myocytes, pancreatic beta-cells and anterior pituitary cells) by routes that are still poorly understood.⁷ Within the cytoplasm of cells, metabolically active iron is present physiologically in low-molecular-weight forms⁸ destined for incorporation into functional compounds or, if present in amounts exceeding cellular requirements, for storage. A cytosolic iron chaperone, poly (rC)-binding protein 1 (PCBP1), delivers excess iron to ferritin,⁹ whose structure maintains large amounts of iron in solution in a compact yet bioavailable form,⁴ diffusely distributed within the cytosol.¹⁰ Iron-free ferritin (apoferritin; Mr 440,000) consists of 24 protein subunits arranged in a spherical shell which can house up to 4,000 atoms in an iron core composed of the hydrous ferric oxide mineral *ferrhydrite* with the approximate formula $5\text{Fe}_2\text{O}_3 \cdot \text{pH}_2\text{O}$.^{4, 11} Recent studies have provided compelling evidence that iron entry and exit from ferritin are the result of an equilibrium based on the concentration of cytosolic iron.⁵ Both iron incorporation and release seem to be intrinsic, autonomous properties of the ferritin molecule.⁵

With low levels of cytosolic iron, soluble iron-containing ferritin is present in the cytosol as randomly dispersed ferritin particles.^{12, 13} As cytosolic iron increases, the concentrations of dispersed ferritin increase and small clusters of ferritin particles appear, still soluble and spread throughout the cytosol. With further increases in cytosolic iron, ferritin is collected in lysosomes by fusion of ferritin clusters with lysosomal membranes, by autophagocytosis, or both.^{6, 12, 13} Digestion of ferritin within secondary lysosomes (siderosomes) leads to denaturation of ferritin protein subunits¹⁴ and to aggregation of the ferritin iron cores, processes thought to result in the formation of amorphous, insoluble masses of hemosiderin.^{12, 13} Under pathological conditions in which the capacity of ferritin to store iron is overwhelmed, ferritin iron may have a pro-oxidant role and contribute to tissue damage.⁴ Production of hemosiderin, enclosed within siderosome membranes, seems to sequester the excess iron away from the cytosol and help protect against iron toxicity. As the total amount of tissue iron increases, the proportion stored as hemosiderin rises, from trace amounts in normal individuals to 90% or more in patients with severe iron overload.¹⁵ Depending upon cellular type, iron supply and utilization, the half-life of cellular ferritin may range from ~20 to 96 hours.¹⁶ Hemosiderin characteristically has a much slower cellular turnover than ferritin.¹⁷ Overall, the available evidence suggests that cytosolic iron is in rapid equilibrium with soluble, dispersed ferritin for short-term storage while exchange with the long-term aggregates of iron within hemosiderin is slow and limited.

The results of recent investigations into the intracellular mechanism of action of iron chelators suggest that measurement of cellular ferritin iron may provide a sensitive means for early assessment of chelator effectiveness. The three iron-chelating agents in clinical use, deferasirox, deferiprone and deferoxamine, all decrease the intracellular concentration of ferritin iron, although by distinct mechanisms. The membrane-permeable iron chelators, deferasirox and deferiprone, were found to cause release of iron from ferritin, followed by monoubiquitination of the iron-poor ferritin and its degradation in the proteasome, decreasing the concentration of ferritin iron.⁶ By contrast, the poorly membrane-permeable iron chelator deferoxamine, a siderophore produced by *Streptomyces pilosus*, was localized within lysosomes, inducing autophagy and then degradation of ferritin within the lysosome, also decreasing the concentration of ferritin iron.⁶ Consequently, measurements of cellular ferritin iron provide a potential means of rapidly monitoring the effectiveness of iron chelators.

EFFECT OF FERRITIN AND HEMOSIDERIN IRON ON MRI SIGNAL DECAY

The differences in solubility and intracellular distribution between ferritin iron (soluble, dispersed and relatively uniformly distributed) and hemosiderin iron (insoluble and aggregated in irregular micron-sized clusters separated by distances of 10 to 30 μm ^{18, 19}) produce different effects on MRI signal decay. Ferritin iron affects MRI signal decay principally through molecular spin-spin relaxation mechanisms,^{20, 21} while hemosiderin aggregates primarily produce magnetic field inhomogeneities that cause spin dephasing.^{22, 23} MRI methods now in use for estimation of tissue iron levels are intended to measure the *total* storage (ferritin + hemosiderin) iron by determining signal intensity ratios or relaxation rates, such as R_2 ($=1/T_2$) and R_2^* ($=1/T_2^*$).^{2, 3} Because the microscopic magnetic field inhomogeneities produced by aggregated iron are very efficient in causing spin dephasing at field levels of 1.5 Tesla and above, these ratios and relaxation rates are predominantly determined by hemosiderin iron and will not, in general, accurately reflect ferritin iron concentrations.

Our approach to the use of magnetic resonance for quantitative determination of tissue ferritin and hemosiderin iron is based on the theoretical model of Jensen and Chandra.^{24, 25} In effect, the physical properties that allow the separation of ferritin (dispersed, soluble iron) from hemosiderin (aggregated, insoluble iron) for biochemical measurements generally correspond to those used in our MRI method. Dispersed, soluble (ferritin-like) iron produces magnetic resonance signal decay with a monoexponential form and the standard transverse relaxation rate R_2 may be derived from monoexponential fitting of a multiple spin echo signal. The R_2 depends linearly on the ferritin iron concentration and increases with the applied field. In contrast, aggregated, insoluble (hemosiderin-like) iron produces magnetic resonance signal decay that is *not* monoexponential and has a strong dependence on the interecho time.^{24, 25} Non-monoexponential MSE signal decay in liver with iron overload has been demonstrated in a number of earlier studies.^{18, 26-28} For determination of storage iron concentrations, a “reduced relaxation rate” (RR_2) and an “aggregation index” (A), are determined by fitting the multiple spin echo signal data to this model.^{24, 25} Subsequently, the ferritin iron concentration can be calculated from RR_2 , and the hemosiderin concentration, from A . Overall, this approach may provide an improved characterization of tissue storage iron and potentially lead to more accurate estimates of the total storage iron concentration. Methods based on a single metric, such as R_2 or R_2^* , have an intrinsic uncertainty resulting from variations in the relative amounts of ferritin and hemosiderin iron.

As more fully described elsewhere,^{24, 25} our approach models the signal decay in iron overloaded tissue by

$$S(t) = S_0 e^{-RR_2 t} \exp \left[-A^{3/4} (\Delta t)^{3/4} (t - t_s)^{3/8} \right], \quad [1]$$

where $S(t)$ is the signal amplitude at a time t , S_0 is the initial signal amplitude, RR_2 is the reduced relaxation rate, A is the aggregation index, and t_s is a time shift given by

$$t_s = 2\tau \left[1 - \left(\frac{\tau}{\Delta t} \right)^2 \right]. \quad [2]$$

It should be noted that Eq. [1] only describes the signal decay at the spin echo times. In addition, when $A = 0$ (i.e., in the absence of hemosiderin iron), the signal decay predicted by Eq. [1] is monoexponential and RR_2 is simply equal to a conventional R_2 relaxation rate.

Our model assumes that the effect of ferritin iron is entirely reflected in the reduced relaxation rate and similarly that the effect of hemosiderin-like iron is reflected in the aggregation index.^{24, 25} The reduced relaxation rate is expected to depend linearly on the dispersed, ferritin-like iron concentration according to

$$RR_2 = r_{2,D} C_D + RR_{2,0}, \quad [4]$$

where C_D is the mass of dispersed iron per tissue mass, $r_{2,D}$ is the dispersed iron relaxivity, and $RR_{2,0}$ is the reduced relaxation rate in the absence of dispersed iron. For the aggregation index our model predicts

$$A = k C_A, \quad [5]$$

where C_A is the mass of aggregated (hemosiderin-like) iron per tissue mass and k is a parameter that depends on tissue properties, including the water self-diffusion coefficient, the mean aggregate spacing, and the applied field strength. If we assume that the parameter k is approximately constant, then Eqs. [4] and [5] imply that the total iron concentration is given by

$$C_T = \alpha_1 + \alpha_2 RR_2 + \alpha_3 A, \quad [6]$$

with

$$\alpha_1 = -\frac{RR_{2,0}}{r_{2,D}}; \quad \alpha_2 = \frac{1}{r_{2,D}}; \quad \alpha_3 = \frac{1}{k}. \quad [7]$$

In addition, we have

$$C_D = \alpha_1 + \alpha_2 RR_2, \quad [8]$$

and

$$C_A = \alpha_3 A, \quad [9]$$

demonstrating explicitly the separate quantification of dispersed and aggregated iron.^{24, 25}

VALIDATION STUDIES OF METHOD WITH PHANTOMS

To validate our approach with known concentrations of dispersed and aggregated iron, phantoms were prepared with different mixtures of manganese chloride (MnCl_2) and iron oxide microspheres designed to mimic the varying amounts of ferritin and hemosiderin found in iron overloaded tissues.²⁵ MnCl_2 was chosen to simulate dispersed iron because it has (longitudinal relaxation time/transverse relaxation time) $T_1/T_2 \approx 10$ at 1.5 Tesla,²⁹ comparable to the T_1/T_2 ratio of aqueous ferritin solutions³⁰ as well as many biological tissues. Another convenient property of MnCl_2 is that the atomic weight of Mn (54.9) is very close to that of Fe (55.8) so that it is reasonable to define, in analogy to tissue concentration, the total iron concentration (C_T) simply as the sum of the Mn concentration (C_D) and the microsphere Fe concentration (C_A). The iron oxide microspheres consisted of magnetite nanoparticles embedded in a polymer matrix (product code UM3CN/5737, Bangs Laboratories, Fishers, Indiana). The absolute iron concentrations used in our phantoms are approximately 100 times smaller than hemosiderin iron concentrations in iron overloaded liver but this is compensated by the fact that the specific susceptibility for the microspheres is about 100 times larger (at static magnetic field $B_0 = 1.5$ Tesla) so that the magnetic effects of the microspheres were similar to those of hemosiderin. The phantoms were imaged at room temperature on a 1.5 Tesla whole-body MR scanner (Intera, Philips Medical Systems, Best, The Netherlands) using three different multi-echo spin-echo sequences. Each sequence had a first echo time at $2\tau = 4$ ms but differing values for Δt (2, 4, and 8 ms), and the final echo occurred at 100 ms in each case. The signal decay data for the even order echoes (i.e., $n = 2, 4, 6 \dots$) were fit globally to the form of Eq. [1] using the Levenberg-Marquardt method.²⁵

Representative signal decay data and fits for the phantom sample bottles are shown in Fig. 1, demonstrating that our approach can model the MSE decay.²⁵ When no microspheres are present, ($C_A = 0$), the MSE decay is nearly independent of the interecho time and essentially monoexponential. By contrast, with microspheres, the MSE decay depends strongly on the interecho time and departures from monoexponentiality are apparent. Figure 2 plots the calculated values for the total, aggregated (hemosiderin-like), and dispersed (ferritin-like) iron concentrations (C_T, MRI , C_A, MRI , C_D, MRI) as functions of the actual iron concentrations, along with the linear regression lines.²⁵ The coefficients of determination are $r^2 = 0.99, 0.99$, and 0.96 , respectively, documenting a close correlation between the calculated and actual iron concentrations. For comparison, neither R_2 nor R_2^* , which are conventionally used for iron estimation, showed a high correlation with the MnCl_2 (ferritin-like) concentration, demonstrating a relative insensitivity to the dispersed component.²⁵

Related MRI studies of phantoms were conducted using a 7 Tesla Bruker MR scanner with (i) uniform agarose gel suspensions of soluble (ferritin-like) superparamagnetic monocrystalline iron oxide nanoparticles and insoluble (hemosiderin-like) superparamagnetic micron-sized particles of iron oxides, and (ii) agarose gel suspensions of iron-loaded cells (mouse embryonic stem cells and human embryonic kidney cells, intracellularly loaded with soluble (ferritin-like) monocrystalline iron oxide nanoparticles or insoluble (hemosiderin-like) micron-sized particles of iron oxides).^{31, 32} For each set of phantoms, a close correlation ($r^2 > 0.98$) was found between the model calculations and the actual iron concentrations.^{31, 32} Altogether, these studies provide compelling experimental validation *in vitro* for our approach to the separate measurement of ferritin and hemosiderin iron *in vivo*.

VALIDATION STUDIES OF METHOD WITH HUMAN LIVER EXPLANTS

To provide further validation for our approach, we have carried out initial studies of samples obtained from diseased livers removed at the time of hepatic transplantation to compare directly our MRI results with biochemical determinations of ferritin, hemosiderin and total storage iron.

³³ In brief, we have examined 18 samples (1.5 cm thick) from 9 human liver explants obtained at transplantation for hepatic failure. Each sample was placed in a 50 mL plastic tube filled with saline and immersed in a cylindrical water bath. To better replicate conditions *in vivo*, the experiment was conducted at 37°C by use of circulating heated water and fiber optic temperature probes. MR scanning was performed with a 5-channel phased array cardiac coil in a 1.5 Tesla Philips MR scanner. Three MSE sequences were acquired with different inter-echo times.³³ A region of interest (ROI) was centered on each of the samples in the phantom and propagated over all the images in the echo train. The non-monoexponential fitting computation was performed using the Marquardt method. After MRI, the liver sections were biochemically analyzed for ferritin, hemosiderin, and total non-heme iron. In these livers, the MR model parameters, RR_2 and A, and the corresponding biochemically determined concentrations of ferritin and hemosiderin were closely correlated ($R \sim 0.9$ for both, $P < 0.0001$)³³ over a range of total storage iron concentrations from 19 to 1,147 $\mu\text{g Fe/g liver}$, wet weight. Studies of additional samples over a wider range of hepatic iron concentrations are needed but these initial results with human liver provide initial evidence of the validity of our MRI method for the separate measurement of ferritin and hemosiderin.

MRI MEASUREMENT OF FERRITIN AND HEMOSIDERIN IRON IN HUMAN SUBJECTS

We initially examined the feasibility of using our MRI method for separate measurements of ferritin and hemosiderin iron using Carr-Purcell-Meiboom-Gill (CPMG) pulse sequences with electrocardiographic triggering and respiratory navigator gating.^{27, 34} Using our MRI method, we determined the total hepatic storage iron concentration as the sum of the ferritin iron and hemosiderin iron. The total hepatic storage iron concentration was within the reference range ($<1.6 \text{ mg Fe/g liver}$, dry weight) in 6 healthy volunteers but increased (range 4.3 to 30.3 mg Fe/g liver , dry weight) in 19 patients with transfusion-dependent thalassemia. The correlation of the MRI-determined total storage (ferritin + hemosiderin) iron with measurements by biochemical analysis of liver samples by biopsy or by biomagnetic susceptometry was 0.95.²⁷

A limitation of the use of such CPMG pulse sequences, or of an alternative, multiple single spin-echo sequences, is that their low data acquisition efficiency generally requires that these studies be performed during free breathing with respiratory gating, making impractical a comprehensive examination of the heart, liver and other organs during a clinically acceptable examination time.³⁵ A recent major advance for the potential clinical application of our method has been the development of multi-echo fast spin-echo pulse sequences that permit accurate R_2 measurement in the heart and liver during a single breath hold, both at 1.5 Tesla³⁵ and at 3 Tesla.³⁶ Studies to provide an initial assessment of the application of these multi-echo fast spin-echo pulse sequences to the detection of changes in myocardial ferritin iron, as yet reported only in abstract form,^{37, 38} have yielded encouraging results. In brief, we examined patients with thalassemia major (10 in New York at 1.5 Tesla; 8 in Hong Kong at 3 Tesla), initially after suspending iron-chelating therapy for one week and subsequently after restarting oral deferasirox (New York) or parenteral deferoxamine with or without oral deferiprone (Hong Kong) for one week. After resuming iron-chelating therapy, our measure of myocardial ferritin iron (mean myocardial RR_2) decreased by approximately 11% ($p < 0.01$) in both sites; R_2^* was not changed significantly.^{37, 38} Further studies in larger numbers of patients with wider ranges of iron loading over longer periods of time are needed but these initial results suggest that measurement of myocardial ferritin iron is a promising investigational method for rapidly evaluating the response of patients with transfusion-dependent thalassemia to new or altered iron-chelating regimens.

CONCLUSIONS

Advances in our understanding of intracellular iron metabolism have clarified the physiological roles of ferritin and hemosiderin. Iron incorporation and release seem to be intrinsic, autonomous properties of the ferritin molecule, with iron entry and exit from ferritin the result of an equilibrium based on the concentration of cytosolic iron. Ferritin can safely sequester large amounts of iron in solution in a compact yet bioavailable form. Nonetheless, under pathological conditions, the capacity of ferritin to store iron may be overwhelmed, with ferritin iron acting as a pro-oxidant and contributing to tissue damage. Production of hemosiderin, enclosed within siderosome membranes, seems to sequester the excess iron away from the cytosol and help protect against iron toxicity. This property may help explain the observation that as the total amount of tissue iron increases, the proportion stored as hemosiderin rises, from trace amounts in normal individuals to 90% or more in patients with severe iron overload. Overall, the available evidence suggests that cytosolic iron is in a rapid equilibrium with soluble, dispersed ferritin for short-term storage while exchange with the long-term aggregates of iron within hemosiderin is slow and limited. The three iron-chelating agents in clinical use, deferasirox, deferiprone and deferoxamine, all decrease the intracellular concentration of ferritin iron, although by distinct mechanisms. Consequently, measurements of cellular ferritin iron provide a potential means of rapidly monitoring the effectiveness of iron chelators.

Established MRI indicators of tissue iron (signal intensity ratios, R_2 , R_2^*) are intended to measure the *total* storage (ferritin + hemosiderin) iron. These ratios and rates are principally influenced by hemosiderin iron, will not accurately reflect ferritin iron concentrations, and change slowly, even with intensive iron-chelating therapy. Moreover, methods to measure the *total* storage (ferritin + hemosiderin) iron based on a single metric, such as R_2 or R_2^* , have an intrinsic uncertainty resulting from variations in the relative amounts of ferritin and hemosiderin iron.

The physical properties that allow the separation of ferritin (dispersed, soluble iron) from hemosiderin (aggregated, insoluble iron) for biochemical measurements generally correspond to those used in our MRI method. The differences in solubility and intracellular distribution between ferritin iron and hemosiderin iron produce different effects on multiple spin-echo signal decay. Dispersed, soluble (ferritin-like) iron produces signal decay with a monoexponential form while aggregated, insoluble (hemosiderin-like) iron produces signal decay that is not monoexponential and has a strong dependence on the interecho time. For determination of storage iron concentrations, a “reduced relaxation rate” (RR_2) and an “aggregation index” (A), are determined by fitting the multi-echo spin-echo decay data to the model of Jensen and Chandra.^{24, 25} Subsequently, the ferritin iron concentration can be calculated from RR_2 , and the hemosiderin concentration, from A . We have validated this approach with known concentrations of dispersed and aggregated iron in agarose phantoms and in initial studies of samples obtained from explanted livers removed at the time of hepatic transplantation to compare directly our MRI results with biochemical determinations of ferritin, hemosiderin and total storage iron.

We first showed the feasibility of using our MRI method for separate measurements of ferritin and hemosiderin iron using Carr-Purcell-Meiboom-Gill (CPMG) sequences with electrocardiographic triggering and respiratory navigator gating. A recent major advance for the potential clinical application of our method has been the development of multi-echo fast spin-echo pulse sequences that permit accurate R_2 measurement in the heart and liver during a single breath hold, both at 1.5 Tesla and at 3 Tesla. Our first studies using the breath-hold multi-echo fast spin-echo pulse sequences have suggested that measurement of myocardial ferritin iron could detect short-term changes produced by iron-chelating therapy. While further studies in larger numbers of patients with a wider ranges of iron loading over longer periods

of time are needed, these initial results suggest that measurement of myocardial ferritin iron is a promising investigational method for rapidly evaluating the response of patients with transfusion-dependent thalassemia to new or altered iron-chelating regimens.

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Figure 1.

Semilogarithmic graph of representative MSE signal decay data (even echoes only) and fits for phantoms consisting of bottles with agarose gels. The data shown are all from bottles having $C_D = 0.0074$ mg Mn/g. The decay for the bottle with $C_A = 0$ (open symbols) is nearly monoexponential and independent of the interecho time. The decay for the bottle having $C_A = 0.04$ mg Fe/mg (solid symbols) is well fit by the form of Eq. [1] with $A = 0.038 \pm 0.001$ $\text{ms}^{-3/2}$, indicating a significant degree of nonmonoexponentiality and interecho time dependence. Reproduced, with permission, from Jensen et al., 2010.²⁵

Figure 2.

MRI measurements for phantoms as functions of C_T , C_A , and C_D together with linear regression lines. (a) $C_{T, MRI}$ found from Eq. [6] with the parameters α_1 , α_2 , α_3 chosen to minimize the residual sum of squares with respect to C_T , resulting in a coefficient of determination of $r^2 = 0.99$. In comparison, (b) R_2 and (c) R_2^* have $r^2 = 0.60$ and $r^2 = 0.92$ with respect to C_T , suggesting that $C_{T, MRI}$ is a better predictor of C_T . Both (d) $C_{A, MRI}$ and (f) R_2^* are strongly correlated with C_A , but (e) R_2 is not. (g) $C_{D, MRI}$ correlates much better with C_D than either (h) R_2 or (i) R_2^* . Reproduced, with permission, from Jensen et al., 2010.²⁵