Quantification of cardiac and tissue iron by nuclear magnetic resonance relaxometry in a novel murine thalassemia-cardiac iron overload model

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OBJECTIVE: To determine whether nuclear magnetic resonance (NMR) relaxation parameters can be used to quantify iron in tissues, the relationship between NMR spectrometric T_2 relaxation measurements and tissue iron concentration were verified in a novel murine cardiac iron overload model.

METHODS: Congenital heterozygous thalassemic mice and controls were injected with intraperitoneal iron or saline and were sacrificed at three weeks. Samples of liver, heart and peripheral muscle were subjected to NMR relaxation measurements and continuous distribution analysis. Tissue ferritin levels were determined with immunoadsorbance techniques, and elemental iron was assayed by flame atomic absorption. Tissues were analyzed pathologically with hematoxylin and eosin and Prussian blue staining to confirm the localization of iron.

RESULTS: This murine iron loading model was uniquely successful in loading iron into the major organs, especially the heart, and produced significant reductions in T_1 and T_2 NMR relaxation values. There was a good correlation between soluble ferritin and total iron levels (r=0.92), indicating that there is a constant and significant fraction of total iron present in ferritin irrespective of absolute iron concentrations. Regression analysis between total iron content and T_2 relaxivity showed a linear relationship (r=0.96), suggesting that the T_2 relaxation parameter is related to tissue iron concentration. The regression relationship suggested that NMR can detect iron levels as low as 0.1 mg/g of tissue.

CONCLUSIONS: Parenteral iron loading in mice produces unique iron overload in major organs, including the heart. Local

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iron deposition is detectable by NMR relaxometry at 0.1 mg/g or higher. There is a linear relationship between iron concentration and T_2 relaxivity. Thus, NMR may be an important and useful clinical tool to quantify iron excess in various pathobiological states of human disease due to iron overload, including heart disease.

Key Words: *Iron, Nuclear magnetic resonance, Restrictive cardiomyopathy, T_2 relaxation*

Quantification du fer cardiaque et tissulaire par relaxométrie en résonance magnétique nucléaire dans un nouveau modèle de thalassémie murine et surcharge ferrique cardiaque

DONNÉES DE DÉPART: Afin de déterminer si les paramètres de la relaxométrie par résonance magnétique nucléaire pouvaient être utilisés pour quantifier le fer tissulaire, les liens entre les mesures spectrométriques de relaxation en T_2 par RMN et la concentration ferrique tissulaire ont été vérifiés dans un nouveau modèle de surcharge ferrique cardiaque murine.

MÉTHODES: Des souris atteintes de thalassémie hétérozygote congénitale et des témoins ont reçu une injection intrapéritonéale de fer ou de solution saline et ont été autopsiées après trois semaines. Des échantillons de tissu hépatique, cardiaque et musculaire périphérique ont été soumis à des mesures de relaxation par RMN et à une analyse de distribution continue. Les taux de ferritine tissulaire ont été mesurés par des techniques d'immunoabsorbance et le fer élémentaire a été mesuré par absorption atomique de flamme. Les tissus ont été analysés sur le plan pathologique par de l'hématoxyline, de l'éosine et un colorant bleu de Prusse pour confirmer la localisation du fer.

RÉSULTATS: Ce modèle de charge ferrique murine a permis une bonne imprégnation ferrique dans les principaux organes, particulièrement le coeur, et a significativement abrégé les temps de relaxation en T_1 et T_2 obtenus par RMN. Une bonne corrélation a été établie entre la ferritine soluble et les taux ferriques totaux (r=0,92), ce qui indique qu'une fraction constante et significative du fer total est présente sous forme de ferritine, peu importent les concentrations ferriques absolues. L'analyse de régression entre le fer total et la relaxation en T_2 a présenté un lien linéaire (r=0,96), ce qui suggère que le paramètre de relaxation en T_2 est lié à la concentration ferrique tissulaire. Le lien de régression suggère que la RMN peut déceler un taux de fer aussi faible que 0,1 mg/g de tissu.

CONCLUSIONS: La surcharge ferrique parentérale chez la souris produit une surcharge ferrique unique au niveau des principaux organes, y compris le coeur. Les dépôts de fer localisés sont décelables à l'aide de relaxométrie par RMN à 0,1 mg/g ou plus. Un lien linéaire s'établit entre la concentration ferrique et la relaxation en T_2. Ainsi, la RMN peut être un outil clinique important et utile pour mesurer l'excès de fer dans divers états biopathologiques chez l'humain dus à une surcharge ferrique, y compris au niveau du coeur.

Iron is a trace metallic element essential for cell metabolism and enzyme function, and its tissue levels are precisely regulated. However, pathological conditions do occur where tissue iron overload either arises congenitally or is acquired through excessive oral or transfusional sources (1-3). Iron overload can be detrimental for cell survival and tissue function, leading to free radical-mediated and other toxic damage to cell organelles (3,4). Indeed iron overload due to chronic blood transfusion for congenital anemias such as thalassemias produces iron-induced heart failure, and is the most common cause of cardiovascular deaths worldwide in the second and third decades of life (3,5).

The available techniques for the assessment of iron in vivo rely on serum chemistry. However, serum iron and ferritin levels do not accurately reflect differential iron storage levels in different organs and are limited by low reproducibility. The only alternative at present is direct tissue biopsy to quantify iron chemically, which unfortunately is invasive and impractical for long term follow-up of chronic conditions.

Nuclear magnetic resonance (NMR) relaxometry is a new diagnostic technique that is sensitive to the presence of iron in tissues, and has the potential to assess iron distribution in patients and to quantify the extent of iron deposition (6,7). This is especially important in the heart, where iron can be preferentially deposited and determine the patient's prognosis. However, the ability to quantify iron requires the establishment of a predictable relationship between tissue iron and unique NMR parameters such as relaxation times. This was limited so far by the lack of either an appropriate animal model of significant multiorgan iron overload or of a large bank of fresh human tissues for correlative studies.

Previous animal models of iron overload have been successful in loading iron into the liver and reticuloendothelial system, but very little in the heart. We have recently developed an improved murine mode of iron overload by parenterally administering iron to strains of inbred mice harbouring a potential beta-thalassemia mutation, leading to differential degrees of tissue iron deposition in major organs, including the heart. By measuring in an NMR spectrometer the T_1 and T_2 relaxation parameters of the freshly removed organs such as the heart, and comparing these values with direct biochemical analyses of tissue iron contents, we can evaluate whether NMR relaxometry represents a new and useful in vivo tool to assess tissue iron stores.

Therefore, the purpose of our study was twofold. First, we sought to establish a novel murine model of iron overload pathologically and biochemically, including cardiac iron deposition. Second, we wanted to determine the relationship between NMR relaxation parameters and tissue iron levels.

ANIMALS AND METHODS

Creation of a murine thalassemia iron overload model: It has been established that a mutation at the beta-globin locus of a DBA/2J male mouse can produce an absolute deficiency of normal beta-major polypeptides in a homozygous mutant. However, homozygous beta-thalassemic mice produce enough beta-minor globin to stabilize the hemoglobin at 8 to

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9 g/dL (8). Hence, beta-thalassemia in mice is a milder disease than that seen in humans, and homozygotes are fertile and are able to propagate this mutation to their progeny.

Heterozygotic progeny from the original DBA/2J x C57BL/6J cross containing the beta-globin mutation were obtained from Jackson Laboratories (Maine). Mice were kept in plastic cages in a well ventilated, temperature-controlled room. F1 generation from the breeding of the original heterozygotic parents provided the animals for this study. The principles of laboratory animal care as promulgated by the National Institute of Laboratory Animal Sciences were strictly observed.

Animals were bled from the retro-orbital sinus, and electrophoresis of cystamine-modified hemoglobin on cellulose acetate was used to distinguish among homozygotic and heterozygotic thalassemic mice and normal wild controls. Thalassemic homozygotes had only Hbd^{minor} ($\alpha_2\beta_2^{dminor}$), homozygous normal mice had only Hb ($\alpha_2\beta_2^{s}$), and heterozygotes had both (9).

Twelve animals were used in this study, comprising two homozygotes, six heterozygotes and four normals. The animals were randomly assigned to be loaded with iron injected peritoneally on an alternate-day regimen (iron dextran injectable, 5 mg/day/animal, approximately 50 mg/week) for three consecutive weeks. The remaining animals were injected with normal saline of equal volume to act as controls. The objective was to produce a wide heterogeneity of iron stores in this cohort of animals for model validation and NMR correlation. At the end of the three-week iron loading period, the animals were sacrificed by decortication. Liver, heart and peripheral gastrocnemius muscle were immediately excised and divided such that one portion was submitted for NMR spectrometry measurements, while the other portion was freeze clamped in liquid nitrogen for future biochemical assessment.

Pathological confirmation of iron deposition: The tissue samples removed were processed, embedded in paraffin block and sectioned for light microscopy. To delineate structure, the sections were stained with hematoxylin and eosin. The endothelial, reticuloendothelial, interstitial and intracellular components were specifically examined in each liver, heart and muscle sample. The same sections were also stained for iron content with Prussian blue.

NMR spectroscopic measurements of tissue relaxation: After sacrifice, liver, heart and peripheral muscle samples were cleared of blood contents, gently blotted, and immediately placed in sealed NMR spectrometer tubes to avoid desiccation and were submitted for NMR spectroscopic analysis of tissue relaxation parameters.

The authors' group has previously characterized in detail the conditions for proper handling of the tissues for NMR spectrometry (10,11). The tissues were examined in a variable field NMR spectrometer (Bruker Model 622S, Germany), at a locked field strength of 1.5 Tesla (proton resonance frequency of 63.9 MHz). This was done to permit ready comparison between the observed relaxation parameters and image derived parameters found in the conventional imaging magnet field strengths of around 1.5 Tesla. All the measurements were made at room temperature of 20±1°C.

T_1 relaxation values were obtained by serial inversion recovery pulse sequences collecting 25 points spanning five relaxation times. The T_2 relaxation values were derived from a Carr-Purcell-Meiboom-Gill pulse sequence incorporating 200 sample points. The pulse widths were tuned for each sample. Typically the transverse relaxation decay curves were adequately modelled with a monoexponential function and were reproducible on repeated measurements to ±3%. The relaxation values were repeated for each specimen and the results averaged. The correlation between the two measured values was excellent at 0.98, and the reproducibility of T_1 and T_2 relaxation times were ±2% and ±3%, respectively.

Biochemical assays for tissue ferritin, iron and protein contents – *Tissue solubilization and preparation:* The collected tissue samples were kept frozen at –70°C until synchronous analysis. The samples were thawed on ice and individually weighed. Tissues were homogenized in freshly prepared Tris buffer containing 0.1 mg/mL phenylmethylsulphonyl fluoride by high energy sonication. After microcentrifugation at 13,600 *g* for 25 mins, the supernatant was analyzed separately as the soluble fraction. The samples were subsequently analyzed both as the total sample for iron content determinations and as the soluble fraction for ferritin content determination.

Tissue ferritin determination: The tissue ferritin levels were calculated from solubilized fraction of the homogenized tissue. Relative ferritin concentration was determined using rat ferritin enzyme immunoassay kits (Ramco Laboratories Inc, Texas). The assay for ferritin depends on an immunoenzymatic reaction, where the primary antibody for ferritin is anchored on a solid phase. The second antibody is conjugated to alkaline phosphatase, which then can react with a colorimetric substrate for quantification. A standard curve was first constructed using rat serum ferritin to characterize the relationship of ferritin concentration versus light absorbance, and this showed an excellent linear relationship (r=0.98). This assay has high sensitivity and a wide response range, and is currently the best technique for assaying a large range of quantities of ferritin. This assay provides mainly a relative measure of ferritin concentration against the ferritin standard used.

Concentrations of various samples were determined in duplicate according to the observed absorbance values. All values were expressed as relative ferritin in mg/g wet weight of specimen (12).

Elemental iron determination: Total iron concentration was measured by flame atomic absorption on an IL 551 Atomic Absorption/Atomic Emission Spectrophotometer (Instrumentation Laboratory, Massachusetts). A high solids air/acetylene burner head and an injection sample cup were employed. A standard curve was also first devised for calibration, before individual samples were submitted for analysis.

Data analysis: The NMR spectrometer relaxation parameters of major organs of liver, heart and muscle between iron

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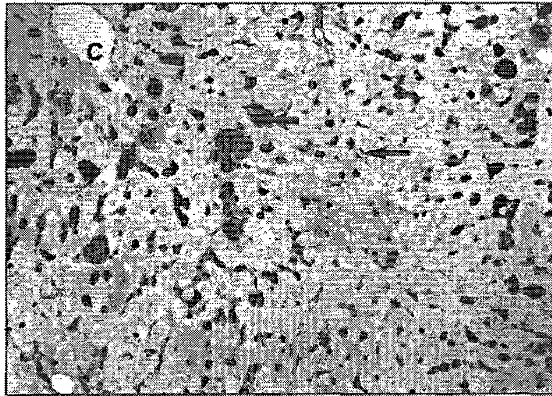

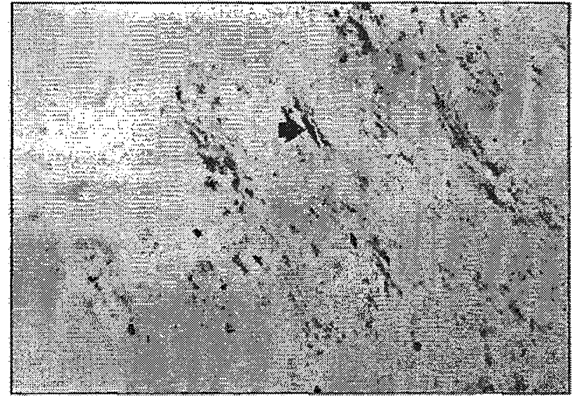

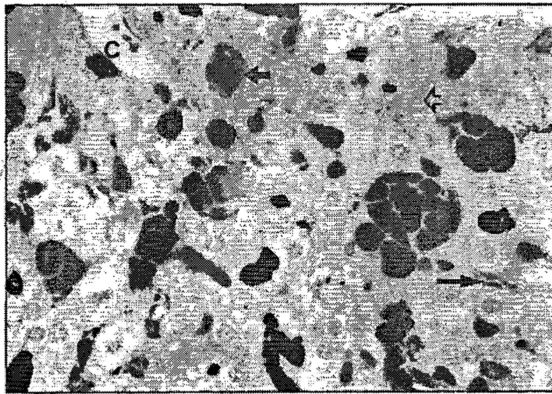

Figure 1) **Above** *Microscopic section of myocardium in a heterozygous animal loaded with iron. The tissue was stained with Prussian blue, and black stained areas represent iron. Abundant Prussian blue positive material (iron) is present, arranged in a linear manner. The heaviest iron deposits are in the endothelial cells (arrowhead). The pale myofibrils contain a patchy fine sprinkling of stained granules diffusely within myocyte cytoplasm. Prussian blue stain, x50.* **Left top and bottom** *Microscopic section of liver from a heterozygous animal loaded with iron. The tissue was stained with Prussian blue, and black stained areas represent iron. Granular iron deposits are seen in virtually all Kupffer cells (dark wide arrowheads) and finer deposits are seen in endothelial cells (dark long arrow). The Kupffer cells show a spectrum of change from 'massive' deposits in clusters to single cells 'bloated' with iron. The cords of hepatocytes contain a fine sprinkling of iron (light arrowhead). C Central vein. Prussian blue stain, x50 (left top) and X125 (left bottom)*

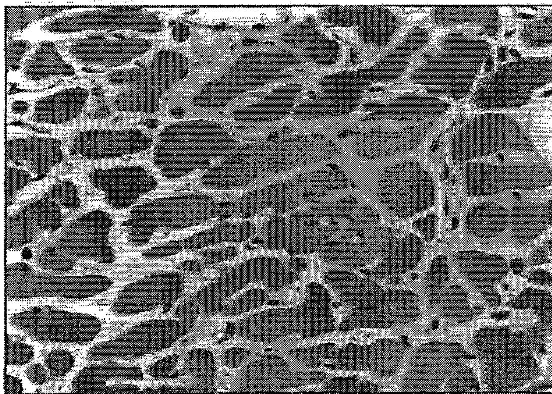

Figure 2) *Microscopic section of myocardium in a heterozygous animal without iron loading. There is no evidence of iron presence in the tissues examined. x99*

loaded and unloaded animals were compared with two-way analysis of variance. Intragroup comparisons were carried out using Neuman-Keul's post-hoc subgroup analysis. Similar statistical procedure was performed for soluble ferritin and total iron concentrations. The relationship between ferritin and iron, and between T_2 relaxivity and iron concentration was determined by linear regression analysis, and the correlation coefficients were calculated. The statistical package used was Statistical Analysis System (SAS Institute, North Carolina), and $P<0.05$ was considered significant.

RESULTS

Thalassemic murine model of iron overload: The model was successful as a model of overloading iron into the organs usually involved in transfusional iron overload. However, the two homozygous animals and some of the heterozygous animals receiving iron injections appeared lethargic with a significant loss of fur colour. All homozygous and the two heterozygous animals with high iron loads had severe weight loss and evidence of 'congestive heart failure' such as paw edema or anasarca. Both homozygous mice died before the sacrifice dates, and the remaining 10 animals were subjected to analysis.

Pathological sections from representative animals were processed with hematoxylin and eosin for structure and Prussian blue stain for iron content (Figure 1). The noniron loaded animals showed normal heart and peripheral muscle architecture (Figure 2). Despite the genetic background in the heterozygous animals, without peripheral iron loading there was no evidence of iron loading in the heart.

On the other hand, in the iron loaded animals, direct iron deposition was observed in the myocyte cytoplasm in the heart. There was also significant iron deposition in the interstitium, bound in endothelial and macrophage systems (Fig-

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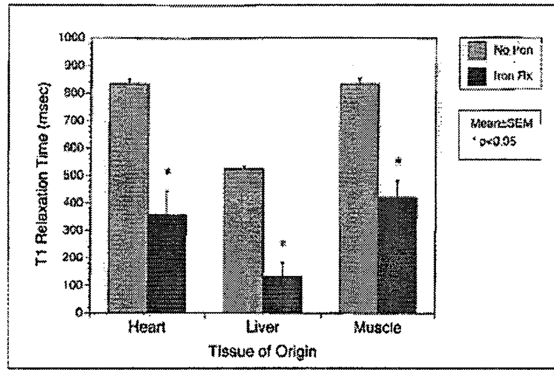


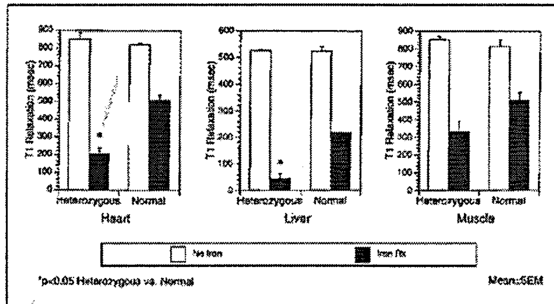
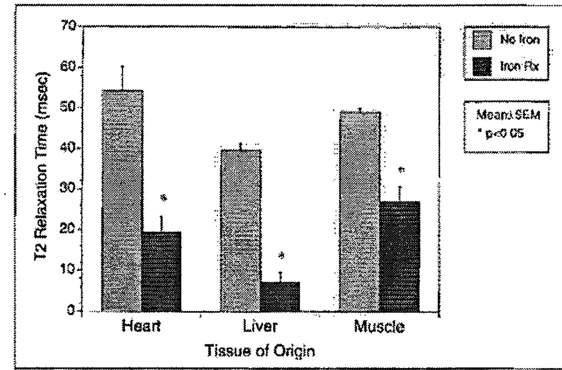


Figure 3) **Top** *Typical T_1 relaxation values representing animals treated with (Rx) or without iron, in tissues from the liver, heart and peripheral muscle. *P<0.05 for iron loading versus no iron loading in the same tissue.* **Bottom** *The same T_1 relaxation times further divided according to animal genotype of heterozygous or normal with respect to globin gene mutation*



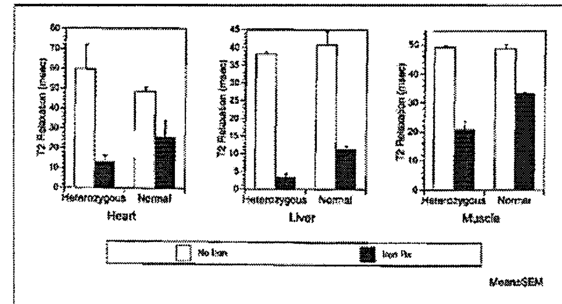


Figure 4) **Top** *Typical T_2 relaxation values representing animals belonging to different iron treatment groups in tissues from the liver, heart and peripheral muscle. *P<0.05 for iron loading (Rx) versus no iron loading in the same tissue.* **Bottom** *The same T_2 relaxation times further divided according to animal genotype of heterozygous or normal with respect to globin gene mutation*

ure 1, left bottom), and within the hepatic Kupffer, endothelial and macrophage cells, with less in the hepatocytes (Figure 1, left top and bottom). The peripheral muscle was relatively free from any intracellular iron, with only occasional Prussian blue stained particles in the interstitium and vascular endothelium. The normal animal with iron loading showed mild iron deposition only in the reticuloendothelial system, but not in the hepatocytes or myocytes. Overall, the liver consistently had more iron than the heart, which in turn contained significantly more than the peripheral muscle.

Spectrometric measurements of tissue relaxation parameters: Representative T_1 and T_2 relaxation values among the treatment groups in the different tissues are plotted on Figures 3 (top) and 4 (top). As expected, the tissues that showed the most dramatic pathological evidence of iron also had the most severe depression of T_2 relaxation, followed by T_1 showing the same trend. Overall, the liver showed the greatest changes, with marked shortening of both T_1 and T_2 values, whereas the heart showed an intermediate amount of iron deposition, and peripheral muscle had minimal level of iron effects. The data comparing the differences according to the genotype of the animals in terms of the globin gene mutation are shown in Figures 3 (bottom) and 4 (bottom). The results suggested that there was more depression of T_1 relaxation in the heterozygous group than in the normal wild genotypes. However, due to the small sample size involved (n=3 for the heterozygotes and n=2 for the normals), statistical analysis is likely not very meaningful, and the results must be interpreted with caution. Overall, iron loading had the most dramatic impact on relaxation changes, followed by the organ involved. The genetic predisposition suggested a secondary permissive effect, which is not quantifiable in this study due to the small sample size involved.

The relaxation decay curves were sufficiently monoexponential to allow extraction of a single relaxation time, particularly for T_1 relaxation. However, the T_2 relaxation decay curves were more complex, containing multiple relaxation components. To gain more insight into the relaxation process in this situation, the T_2 decay curves could be examined using continuous distribution analysis of relaxation times (13). Using this analysis technique, the specific T_2 components can be identified, and their relative strength of contribution to the relaxation process can be compared between experimental groups. Typical T_2 distribution analysis for the liver from normal and heterozygous animals with or without iron loading are shown in Figure 5. The normal control had a dominant contribution from a peak with a mean T_2 value of 40 ms. The iron loaded normal mouse showed a bimo-

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