Akademie věd České republiky

Teze doktorské disertační práce
k získání vědeckého titulu "doktor věd"
ve skupině věd molekulárně biologických a lékařských

NMR AND MRI RELAXOMETRY OF PARAMAGNETIC COMPOUNDS AND IRON-STORAGE PROTEINS. IMPLICATION TO CLINICAL MEDICINE

Komise pro obhajoby doktorských disertací v oboru biomedicína

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Souhrn

Předložená práce se zaobírá problemtikou detekce a možné kvantifikace nehemového mozkového železa a ostatních paramagnetických iontů a sloučenin, které mohou hrát úlohu v procesu stárnutí a rozvoje některých (zvláště neurodegenerativních) onemocnění.

Práce sleduje stejný pochod, jaký prodělala původně experimentální nukleární magnetická resonance (NMR) k své medicinské klinické aplikaci – magnetické rezonanci (MRI): časově prvé předložené práce měří pomocí NMR relaxometrie jednoduché chemické sloučeniny paramagnetických kovů a metalloproteinů ferritinu a hemosiderinu. Pozdější práce již zkoumají "*ex vivo"* vzorky mozkových tkání, známé především bohatým zastoupením železa. Závěrečné práce pak aplikují nabyté poznatky ze základního výzkumu na klinická data získaná u zdravých dobrovolníků i pacientů, trpících neurologickými onemocněními.

Předložený soubor prací přináší některé prioritní výsledky: poprvé byl systematicky pomocí relaxometrie dokumentován efekt tzv. kontaktních interakcí na T2 relaxační časy některých paramagnetických kovů. Byla vyvrácena teorie o kvadratické závislosti T2 relaxační rychlosti (1/T2) ferritinu na magnetickém poli a proto není aplikovatelná teorie chemické výměny k vysvětlení magnetických vlastností tohoto metaloproteinu. Lineární závislost T2 relaxační rychlosti na síle magnetického pole, dosud nepodložená validní fyzikální teorií, byla prokázána pro ferritin, hemosiderin, mozková bazální ganglia s vysokým obsahem ferritinu i arteficielní oxidy železa, které se později staly dostupnými kontrastními látkami pro klinickou magnetickou rezonanci.

Měření pomocí in vivo MR relaxometrie u pacientů s Parkinsonovu chorobou a některými typy tzv. Parkinson plus syndromů poukázalo na pravděpodobnost snížení hladin ferritinu v mozkové kůře. Se zvýšením hladin nízkomolekulárního železa by docházelo k vyššímu oxidativnímu stresu a dle posledních poznatků by tak mohlo dojít i ke zvýšené prodkukci glutamátu, který je podezříván z přímého pategenetického působení u některých neurodegenerativních onemocnění.

Měření u pacientů trpících jaterní cirhosou prokázala zvýšenou akumulaci pravděpodobně manganu v bazálních gangliích. Signální charakteristiky i relaxační časy se vrací k normálu po úspěšné jaterní transplantaci.

Autor této disertace pokračuje v klinické aplikaci výzkumu za grantové podpory u Huntingtonovy chorey a amyotrofické laterální sklerozy.

Introduction

This dissertation thesis is focused on detection and possible quantification of brain iron and other paramagnetic ions and compounds using nuclear magnetic resonance (NMR) and magnetic resonance imaging (MRI). Iron is the most abundant paramagnetic element in the human brain (1). It is believed that it plays an important role in the process called oxidative stress and thus may be crucial in some changes that occur in the process of aging and also in various neurological (especially but not exclusively neurodegenerative) diseases.

Iron is present in the brain in basically two forms: heme iron in hemoglobin and some iron-containing enzymes and non-heme iron. Non-heme iron exists in various forms such as high-molecular weight ferritin, hemosiderin or transferrin and low-molecular weight forms of iron (2,3). The presented work will mainly focus on non-heme forms of iron in ferritin and hemosiderin.

Ferritin is a ubiquitous metalloprotein, present in species from microbes to humans. It is water-soluble. The main function of ferritin is storing iron in the biologically available and non-toxic form. One molecule of apoferritin (protein with no iron) is able theoretically to bind up to 4 500 atoms of iron (loading factor) (4). Hemosiderin is a water-insoluble degradation product of ferritin. It is believed that hemosiderin is present in higher concentrations in the diseased brain (5) while ferritin accumulates in the brain both in the physiological process of aging and also in disease (1,5).

Other paramagnetic substances such as e.g. copper or manganese may accumulate in the brain under some pathological conditions. As shown later a number of paramagnetic substances were measured using NMR relaxometry.

This thesis follows the same pattern as NMR did in its transition into MRI: The first presented projects (NMR) show the results of basic research performed with an experimental equipment – T1/T2 relaxometer – on simple chemical solutions of iron and other paramagnetic ions. Following projects correlate those results with „*ex vivo*“ samples measured with the same instrument. Finally most recent work (MRI) shows the

implication of the theories and results on *in vivo* scanning of healthy volunteers and patients suffering from various neurological diseases.

The experimental part of the work was performed at the National Institutes of Health (NIH), Bethesda, Maryland, USA, Albert Einstein College of Medicine, New York, USA and Hebrew University, Jerusalem, Israel. The clinical part was performed at NIH, University of Milano, Italy and at the Institute of Clinical and Experimental Medicine, Prague, Czech Republic.

The goal of this dissertation thesis is not only to show the scientific data, its analysis and implications. The author of the dissertation thesis would also like to point out the important link between basic and clinical science as a fundamental feature of modern research.

Methods

The "NMR" part of the project was performed with T1-T2 relaxometer. This unique instrument uses a small electromagnet that produces external magnetic field strengths from 0.04 (later 0.02) to 1.5 Tesla with corresponding Larmor frequencies between 2 (later 1) and 65 MHz. T1 relaxation times are measured with saturation recovery technique with 32 incremental data points. T2 relaxation times are obtained using a Carr-Purcel-Meiboom-Gill (CPMG) sequence. An echo train following a 90 pulse may vary from 2 to 500 data points with variable interecho spacing. A stable temperature between ca 0° C and 40° C can be maintained during the measurements.

This is a non-imaging system that is able to measure solutions or tissue samples of ca 1 cc volume, placed in a test-tube. When the samples are not measured they are kept in a pre-heated holder or cooler in order to maintain a stable temperature. The technique is described in detail elsewhere (6).

The MRI part of the project was performed on patient MR scanners with external field of 1.5 and 4 Tesla. T1 relaxation time was measured with saturation recovery technique with TR times of 100, 200, 400, 600, 1000

and 1500 ms. T2 relaxation time was measured with a single slice CPMG sequence with interecho times 22, 40, 60… 320 ms. The repetition times was 2 000 ms. T1 was calculated using an exponential curve fit with six points. T2 was determined from T2 maps. ROIs were placed in the basal ganglia, extrapyramidal brainstem nuclei, white matter and cerebral cortex. (5).

The expected iron concentration for given age and ROI were determined using measurements performed by Hallgren and Sourander (1). The iron concentration was determined spectrphotometrically or using Mossbauer spectroscopy (7).

NMR relaxometry of paramagnetic ions

The T1-T2 analyzer was the first instrument capable of measuring T2 relaxation times over a wide range of magnetic fields. Until that time, the so called field-cycling relaxometers measured only T1 relaxation times over a range of magnetic fields and the T2 measurement was performed only at one given field strength.

We measured T1 and T2 relaxation times of water solutions of copper, gadolinium, manganese, iron, chromium and dysprosium, both in free and chelated forms. The results were published in Journal of Magnetic Resonance Imaging (8). We showed (Fig.1) the effect of the so-called contact interactions on T2 relaxation for non-chelated solutions of iron (Fe^{3+}), manganese and chromium. The contact interaction does not affect T1 at MR imaging fields but shortens T2. It is due to isotropic interaction between the electron spin and protons in the first hydration shell.

We also showed that due to its small magnetic moment, the relaxation times of copper are low. Thus, in copper-storage disease such as Wilson disease, the direct effect of accumulated copper on the resulting signal intensity is usually negligible.

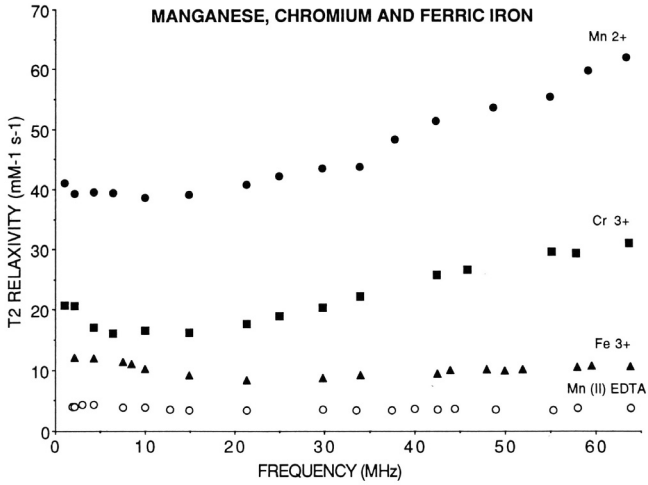


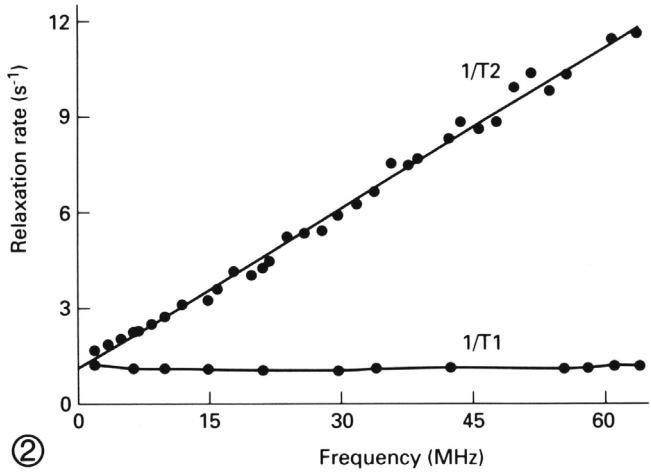
Fig. 1

Basic magnetic properties of ferritin and similar artificial substances (iron oxides)

We decided to study the magnetic properties of a ferritin molecule in a detailed systematic way – from NMR *in vitro* experiments into *in vivo* MRI scanning. We started our projects with detailed measurements of T1 and T2 relaxation times over a wide range of magnetic fields (0.02 Tesla – 1.5 Tesla).

It was assumed that ferritin would behave in a similar way as e.g. deoxyhemoglobin (9) as it was predicted by two-site chemical exchange equation (9,10). This equation deals with two fractions of water molecules distributed close and far to the molecule of interest. It takes a given time for the molecules to "exchange" or to "diffuse" between the two sites. The theoretical formula (see the paper) assumes a quadratic dependence on ΔB. ΔB implies for a difference between two local magnetic fields (that cause by the magnetic particle and that not influenced by it).

However, this initial study (6) brought a new *unexpected* and still *unexplained* observation: T2 relaxation rate of medium-loaded metalloprotein ferritin increases linearly between 0.02 Tesla and 1.5 Tesla (Fig. 2). Our later (and unfortunately unpublished) data proved the same effect up to 7 Tesla. We observed a similar linear dependence in artificial ferritin-like substances (11). Some of them proved to be new promising T2 contrast agents for MRI. We concluded that the linear dependence is a



unique property of a ferrihydrite core. Such substances became later pivotal for synthesis of new T2 contrast agents (12).
Fig. 2.

Almost everything in the nature behaves in a non-linear way. Thus, such a linear effect – we believe that the linear dependence in this case is an upward part of a later-saturation curve – had no relevant theoretical explanation and until now has not been supported by an accepted physical theory.

These unexpected and unexplained findings led us to investigate the molecule of ferritin in a more detailed way. Results were later published in Journal of Inorganic Biochemistry (13) and Magnetic Resonance in Medicine (4,14).

We studied detailed magnetic properties of a ferritin molecule loaded with various amounts of iron atoms from 0 (apoferritin) to 3400. The ferritin samples with low loading factors also brought some unexpected results and broke down some established theories about iron loading into the apoferritin. First ca 13 iron atoms cause the initial increase of relaxivity (both T1 and T2). This is consistent with the accepted and expected model entailing binding and oxygenation of Fe^{2+} at specific iron sites on the protein shell. However, T1 and T2 relaxivities of samples with loading factors between ca 14 and 50 showed a decrease of relaxivity. This finding was unexpected and a new theory was established. Such a reduction of T1 and T2 relaxivities may be consistent with the formation of antiferromagnetic dimers which cause mutual cancellation of Fe^{3+} spins. In other words, if the binding site binds only one atom of iron the resulting paramagnetic effect of Fe^{3+} causes T1 and T2 shortening and thus increase in relaxivities. However, if two Fe^{3+} atoms bind to a given site, due to the formation of dimers, the magnetic effect is cancelled and the relaxivities are reduced (Fig 3).

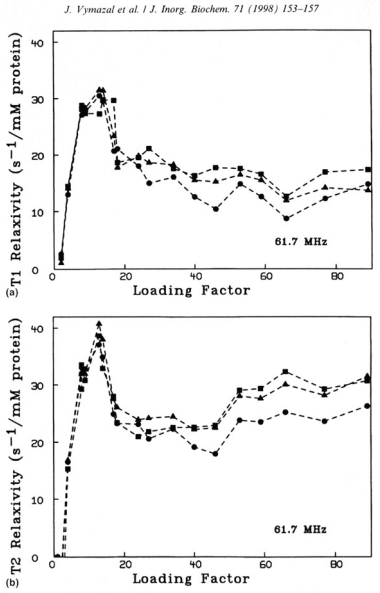


Fig.3

With loading factors of ca 70 and more the ferrihydrite core is formed, causing preferential increase in T2 relaxivities that is proportional to the field strength (Fig. 4).

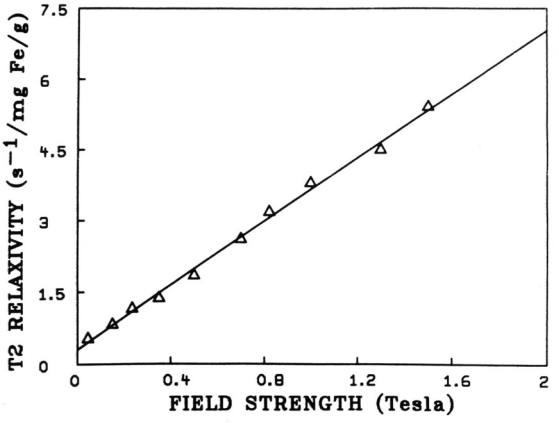


Fig. 4

Magnetic properties of blood. Good news: the chemical-exchange theory works at least for deoxyhemoglobin

The chemical exchange theory completely failed to explain relaxation mechanisms of ferritin. We wanted to test whether this theory is applicable for T2 relaxation times of deoxygenated blood. Deoxyhemoglobin had been known to be paramagnetic. These results were published in Journal of Magnetic Resonance Imaging (15).

The oxygenated blood showed almost no dependence on external magnetic field, except for some protein effect at very low magnetic fields. Deoxygenated blood, on the other hand, showed a reliable quadratic dependence of T2 relaxation rates (Fig 5) as it was predicted by two-site

chemical exchange formula developed originally for the exchange of protons between two different chemical sites.

$1/T2 = R_o + R_{max}[1/(\tau_{ex}/\tau)\tanh(\tau/\tau_{ex})]$

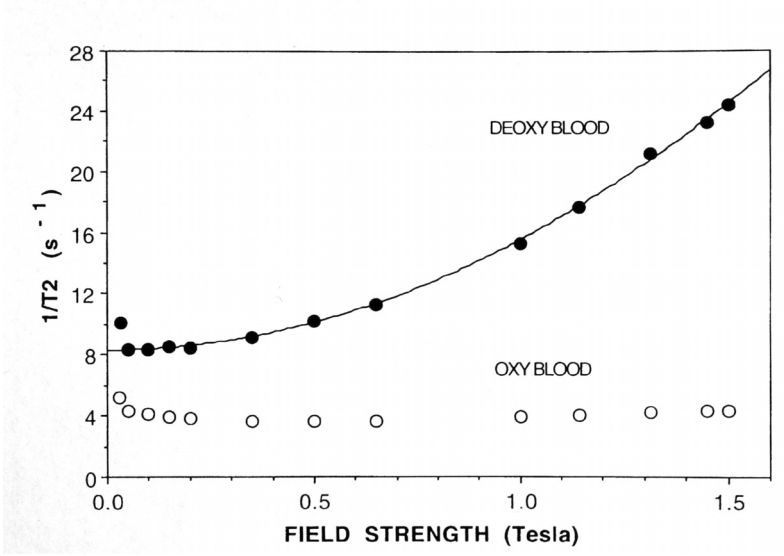


Fig. 5

where R_o is the relaxation rate (1/relaxation time) in the absence of diffusion and R_{max} is the maximum diffusion-induced relaxation rate. Thus τ_{ex} becomes a measure of the diffusion time through the magnetic disturbance caused by the presence of paramagnetic substance.

The chemical exchange formula is also applicable to the dependence on NMR echo time (TE) (Fig 6).

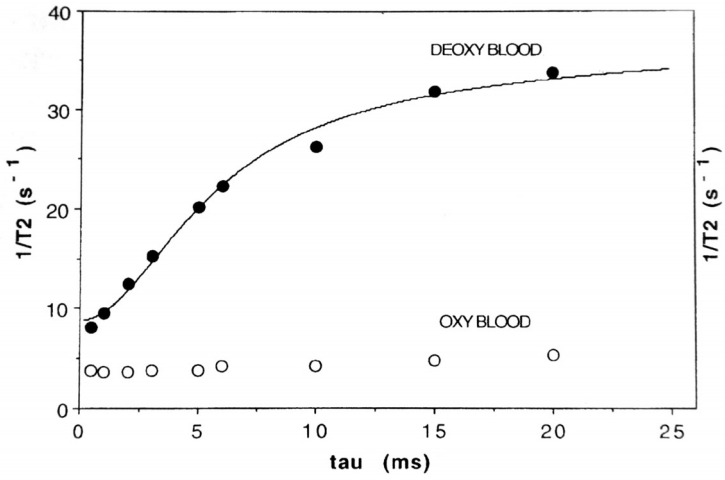


Fig. 6

Magnetic properties of extrapyramidal nuclei – comparison with ferritin and *in vitro* studies

Extrapyramidal brain nuclei (caudate, putamen, pallidum, subst. nigra, ncl. ruber) contain a significant amount of iron (1). Our measurements of caudate and pallidum samples show a similar linear field dependence as our previous data on ferritin (Fig. 7). We consider this field dependence to be a “footprint” of ferritin and similar molecules.

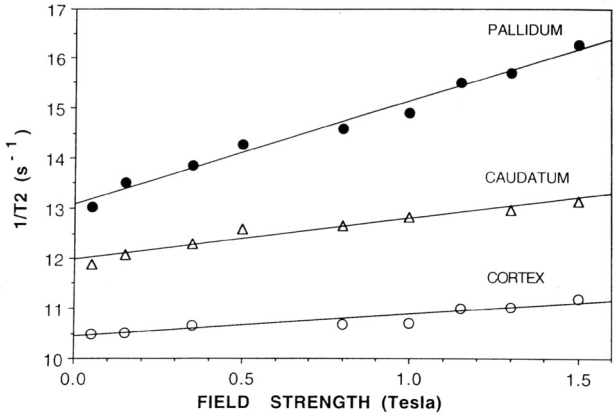


Fig 7

We also observed a dependence on (inter)echo time (Fig. 8), suggesting that ferritin in tissue is not homogenously distributed (as it is in solution) but rather clustered causing inhomogeneities of the local magnetic field and thus further shortening of T2 (T2*). On the contrary we did not observe any dependence on echo time in ferritin solution where molecules of ferritin are homogenously distributed within the solution.

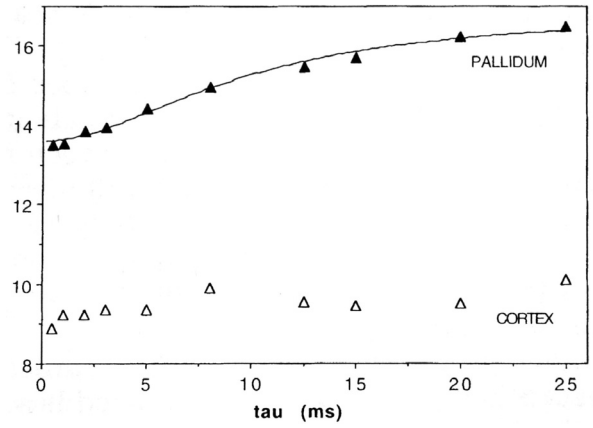


Fig. 8

Later we measured more iron/ferritin-containing samples from more nuclei and also from cerebral cortex and white matter. A sample of cavernous hemangioma was also measured. Cavernous hemangiomas are known to contain high amounts of hemosiderin-bound iron. Hemosiderin is degradation product of ferritin that is less organized and water insoluble therefore cannot be measured as ferritin in a solution. The paper was published in Magnetic Resonance in Medicine (16).

Brain samples containing high amounts of iron (especially the globus pallidus – closed circles on Fig.9) showed a similar linear dependence between the T2 relaxation rate and magnetic field strength (Fig. 9). A cavernous hemangioma sample (closed squares on Fig. 9), known to accumulate iron in the form of hemosiderin, showed a similar linear dependence.

An important step forward was the fact that we were able to measure the total iron content in most of the samples and in some of them, we tried to estimate the form of the iron using Mossbauer spectroscopy (7). Mossbauer spectra, performed at Hebrew University in Jerusalem, proved that at least 80% of iron in the globus pallidus is in the form of ferritin. We consider this result to be an important moment to establish a theory that ferritin is the main contributor for the contrast observed within the physiological gray matter on MRI. Thus this paper in certain sense bridges the gap between *in vitro* and *in vivo* data.

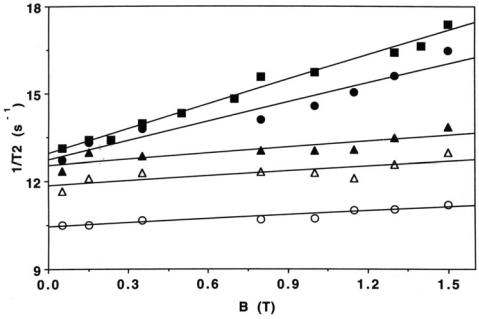


Fig. 9

In vivo detection of brain iron with MRI

After some retrospective studies (17) where we measured signal intensity ratios, we measured a number of healthy volunteers and also patients suffering from Parkinson´s disease and Multiple system atrophy (MSA) - the so-called Parkinson plus syndrome with quantitative MRI. The patients and volunteers were scanned at University of Milano, Italy.

We measured both T1 and T2 relaxation times in a systematic way. The results were published in Radiology (5). In a relatively large number of patients with Parkinson´s disease and MSA, we were able to prove that the patients suffering from MSA show a shortening of both T1 and T2 relaxation times in the globus pallidus in comparison with healthy age-mateched volunteers. These changes can be attributed to the accumulation of ferritin. Both patients suffering from Parkinson´s disease and MSA showed changes that might be attributable to *decreased* ferritin levels in the cerebral cortex. "Free", non-chelated iron is known to be toxic because it can take a part in the enhancement of the oxidative stress. The incorporation of iron into protein may have a protective effect. Thus a decrease of ferritin levels in the cerebral cortex may be in agreement with the accepted theories of the role of oxidative stress in the pathogenesis of Parkinson and similar diseases.

Recent data give our projects a new dimension: it was proved that iron alters glutamate secretion by regulating cytosolic aconitase activity (18). Glutamate has many important physiological functions including its role as a neurotransmitter in the retina and CNS. It seems that changes in the iron levels directly influence glutamate production. Indeed, glutamate alterations were found in diseases such as Parkinson´s disease, Huntington chorea etc. where changes in the iron/ferritin levels occur (19). Thus, changes in the iron levels in the so-called neurodegenerative diseases may have further impact not only on oxidative stress but also on brain the metabolism of some brain neurotransmitters.

The author of this dissertation thesis was invited to write two review articles devoted to the problematic of brain iron. The papers were published

in Journal of Neurological Sciences (20) and in Cellular and Molecular Biology (21).

In vivo detection of other paramagnetic compounds

Particular signal changes were observed in the brains of patients suffering from chronic liver disease or on parenteral nutrition. The signal intensitiy in the basal ganglia, especially in the globus pallidus is increased on T1-weighted images. The fact that the increased signal intensity on T1-weighted images may be transitory and e.g. after successful liver transplantation disappears, makes this topic even more interesting.

Thus we decided to collect quantitative T1 and T2 data from the gray and white matter regions from patients suffering from hepatic cirrhosis and from age-matched healthy controls.

This project was performed in the collaboration with Institute of Clinical and Experimental Medicine in Prague. The following paper was published in American Journal of Neuroradiology (22).

We proved that the observable T1 changes (Fig. 10A) are not the only detectable changes. The T1 effect is followed by the somewhat hidden T2 shortening (Fig 10B). In fact any shortening of T1 relaxation time should lead to an adequate T2 shortening (the opposite is not true).

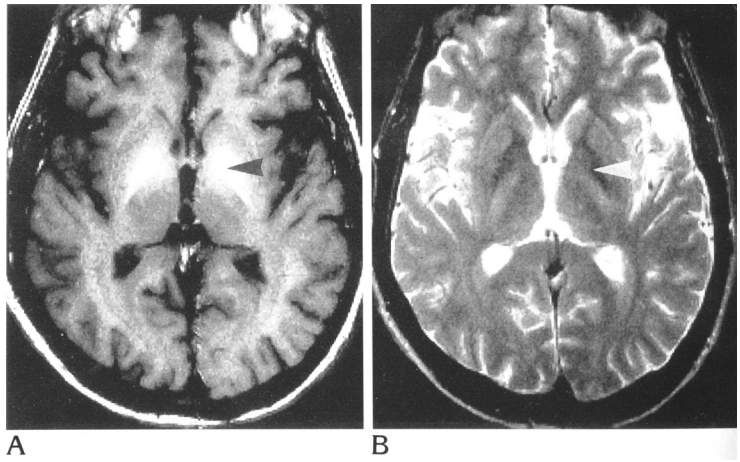


Fig. 10

The T1/T2 ratio of the accumulated agent does not have a ferritin "footprint". Our results are in agreement with the theory that the accumulation of manganese may be responsible for the observed changes on the T1-weighted image (and quantitatively detectable on the T2-weighted image). Since it has been known that the above described changes may decrease or disappear after successful treatment, we decided to extent our project and quantitatively measure the patients suffering from the hepatic cirrhosis before and after the liver transplantation. The data were published in MAGMA (23).

Our quantitative data confirmed the transitory character of the T1- and T2-shortening. This observation confirms that gross morphological changes cannot be responsible for such signal intensity modifications. On the other hand the data support the idea of accumulation of a paramagnetic agent. Thus we believe that manganese is responsible for such changes and due to its magnetic properties (4) can be detectable *in vivo* using MRI. These changes are contrary to those observed in Wilson disease, where copper is accumulated. However the signal intensity changes are primarily governed by gliosis and not by copper itself because its magnetic moment is

too low for *in vivo* MRI detection. Thus it would be more difficult to monitor a treatment success using *in vivo* MRI because morphological changes occur.

Conclusion

MRI has become an important clinical and research tool for non-invasive imaging. Electromagnetic energies used in MRI are unable to break the chemical bond thus MRI does not have a radiation burden. In order to better understand *in vivo* MRI of various diseases it is important to have a solid theoretical basis for the observed signal intensity changes, supported by physico-chemical theories and *in vitro* experiments.

Magnetic properties of various forms of iron and other paramagnetic ions were presented in this dissertation thesis. A correlation between *in vitro* and *in vivo* data was introduced.

We believe that iron is the main contributor to the gray matter contrast in the healthy (and also aging) brain. We proved that several forms of iron can be distinguished with *in vivo* MRI and that some other paramagnetic ions can be detected in the same way. Our results give a basis for *in vivo* quantification of the amounts of iron or another paramagnetic ion.

Recent data proved that iron may alter glutamate levels. Glutamate has many important physiological functions including its role as a neurotransmitter in the central nervous system. It seems that changes in the iron levels directly influence glutamate production. Thus, changes in the iron levels in the so-called neurodegenerative diseases may have further impact not only on oxidative stress but also on brain the metabolism of some brain neurotransmitters.

Our research projects continue: currently we have been performing a prospective thorough study on relaxometry and volumometry in patients suffering from Huntington chorea and age-matched controls. This study is supported by the grant IGA MZ ČR NF/7623-3/2003. A similar project in patients suffering from motor neuron disease has recently been approved as IGA MZ ČR NR/8491-3/2005.

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SCI related to the topic: **325**
SCI of the author until March 3, 2005: **417**