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MR of Human Postmortem Brain Tissue: Correlative Study between T2 and Assays of Iron and Ferritin in Parkinson and Huntington Disease

Julian C. Chen,[1] Peter A. Hardy,[4] Walter Kucharczyk,[1,5] Martin Clauberg,[3] Jayant G. Joshi,[3] Anastasios Vourlas,[1] Madhu Dhar,[3] and R. Mark Henkelman[2]

PURPOSE: To test the hypothesis that the T2 shortening observed on MR images of the brain in patients with Parkinson and Huntington diseases is due to tissue iron deposition. **METHODS:** Tissue iron and ferritin assays were performed on postmortem putamen and globus pallidus samples from subjects with Huntington and Parkinson disease. The assays were correlated with T2 measurements. Normal samples were included as controls. **RESULTS:** There were significant differences in T2 values, and iron and ferritin concentrations among the putamen samples. Compared with normal controls, subjects with Huntington disease had approximately a threefold increase in iron and a sixfold increase in ferritin concentrations; however, they also had the ***longest*** T2 values. Parkinson disease putamens had milder elevations of iron concentrations above that of controls (33%) and demonstrated slightly shorter T2 values. The globus pallidus samples demonstrated a similar trend in their T2s, iron, and ferritin levels, but there was a larger overlap in the T2 values. **CONCLUSIONS:** Our results indicate that tissue iron and ferritin concentrations are elevated in the brains of subjects with both Parkinson and Huntington disease but the elevated concentrations do *not* correlate with T2 shortening. Although iron and ferritin can shorten T2, we conclude that other factors must play a significant role in determining the T2 relaxation time and that iron or ferritin are not dominant in this regard.

Index terms: Huntington disease; Parkinson disease; Basal ganglia; Neuropathology

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Many investigators have sought to demonstrate a correlation between tissue iron concentration and T2 relaxation to support the hypothesis that storage iron causes regional hypointensity on T2-weighted magnetic resonance (MR) images. Some studies have found evidence to support this hypothesis (1–6), while others have not (7–10). We have previously reported a study that showed no correlation between tissue iron concentration and T2 relaxation at the concentrations of iron encountered in the normal brain (7). While we did not dispute the fact that iron causes T2 shortening, or that iron is responsible for reduced signal intensity in some tissues, we concluded that the amount of iron present in *normal* human brain tissue was not the major determinant of T2. Brain tissues with abnormally high amounts of iron were not included at that time.

The purpose of this study was to measure and correlate T2 relaxation with iron and ferritin concentration in postmortem brain tissue of subjects with Huntington and Parkinson disease. Brain tissue from subjects with these disorders is known to have higher than normal concentrations of iron (11–14).

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[1] Department of Radiology, University of Toronto, Toronto, Ontario, M5S 1A8, Canada.

[2] Department of Medical Physics, University of Toronto and Sunnybrook Health Science Centre, Toronto, Ontario, M5S 1A8, Canada.

[3] Department of Biochemistry, University of Tennessee, Knoxville, Tennessee, 37901.

[4] Department of Diagnostic Radiology, Cleveland Clinic Foundation, 9500 Euclid Avenue-Desk L10, Cleveland, Ohio, 44195–5128.

[5] Address reprint requests to: Walter Kucharczyk, M.D., F.R.C.P. (C), Department of Radiology, University of Toronto, Room 127 Fitzgerald Building, 150 College Street, Toronto, Ontario, M5S 1A8, Canada.

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Materials and Methods

Specimens

The brain tissue used in this investigation was provided by the Canadian Brain Tissue Bank. The age of the subjects ranged from 19 to 90 years. There were 13 male and nine female subjects. The subjects included normal controls (n = 6), Parkinson disease (n = 10), and Huntington disease (n = 6). All tissue was handled in a uniform and consistent fashion. No fixative or preservative agents were used at any time. Following death, the brains of subjects were removed intact from the cranium and stored at −60°C for 1 to 6 months in sealed containers. The brains were then thawed for excision of sample specimens from the putamen and globus pallidus. The specimens were sealed in airtight 1-mL vials to prevent dehydration. The containers were opened only for biochemical analysis.

NMR Spectrometry

The T2 values of the samples were measured at 25°C with a home-built 1.5-T spectrometer based on a B-E 40A5 resistive magnet (Bruker Medical Instruments, Billerica, MA) with a variable power supply set to provide a field of 1.5 T. A Carr-Purcell-Meiboom-Gill sequence, with a minimum of 25 echoes spanning five relaxation times was used with a TE = 2.5 msec. Repeat measurements with TE = 0.75 msec did not differ by more than 4%. The spatial inhomogeneity of the magnet over the sample is less than one part per million. The T2 measurements were reproducible to ± 3% and accurate to within 10%.

Biochemical Assays

The brain specimens were weighed and homogenized by two cycles of low-energy sonification in five times their volume of 0.1 mol/L of tris(hydroxymethyl)aminomethane-hydrogen chloride buffer (pH = 7.4) containing freshly prepared 0.1 mg/mL of phenylmethyl-sulfonyl fluoride to yield crude homogenate. One-milliliter aliquots of the crude homogenate were spun at 13,600 *g* for 25 minutes at 4°C. The supernatant (soluble) fraction and the crude homogenate were assayed for both iron and ferritin content. Protein was determined in the soluble fraction. All measurements were performed in duplicate. Iron concentration was measured with a flame atomic absorption spectrophotometer (IL 551 Atomic Absorption/Atomic Emission Spectrophotometer; Instrumentation Laboratory, Wilmington, MA). Ferritin concentration was measured using a commercially available radioimmunoassay kit (Catalog #F-11 Ramco Laboratories Inc, Houston, TX) that was calibrated to internal standards provided by Ramco Laboratories Inc. The soluble fraction iron represents those iron species that can be isolated with ferritin, whereas the crude fraction iron would include all other forms of iron such as hemosiderin and other organic as well as inorganic iron species.

Results

Iron and Ferritin Assays

The methods for determining iron and ferritin concentrations in this experiment were identical to those used in a previous investigation (7). The assay for iron in normal controls yielded consistent results between the two investigations with no statistical difference. However, the assay for ferritin was inconsistent between the two studies, the current investigation yielding results approximately one order of magnitude greater than our previous study.

A detailed review of our methodology suggested that the source of error was inherent in the radioimmunoassay for ferritin arising from lack of a brain specific monoclonal antibody (see Results-Sources of Error). Because of the inconsistency between the two studies, we quantitated the tissue ferritin levels as relative values with respect to the mean of the normal control values. Hence the latter has been standardized to a numerical value of 1.0. On the other hand, iron assays, analyzed by flame atomic absorption spectrophotometry were consistent between the two studies. Therefore, we have used their absolute values in reporting our current data.

Putamen (see Table 1)

The T2 values and the iron and relative ferritin concentrations in putaminal tissue were statistically different (confidence level > 95%) for normal controls, Parkinson disease subjects, and Huntington disease subjects. The longest mean T2 and the highest mean iron level (197 μg/g, crude fraction), as well as a sixfold increase of ferritin, were found in Huntington disease subjects. In Parkinson disease subjects, there were significantly higher iron levels (soluble and crude), as well as a 1.7-fold increase of ferritin. Parkinson subjects had a mean T2 value significantly shorter than the normal group ($P < .05$). There was no

TABLE 1: Putamen: T2, iron, and ferritin

	T2 (msec)	Iron, soluble (μg/g)	Iron, crude (μg/g)	Ferritin (μg/g)
Normal (n = 6)	78 ± 3	35 ± 6	75 ± 10	1.0 ± 0.2
Parkinson (n = 10)	73 ± 4	52 ± 15	99 ± 20	1.7 ± 0.9
Huntington (n = 6)	85 ± 7	116 ± 33	197 ± 54	6.0 ± 3.6

Note.—The soluble and crude iron levels are expressed in micrograms per wet gram of tissue (μg/g). The ferritin values are numerical ratio of the mean control (normal) values in putamen or globus pallidus. The T2 values are in milliseconds (msec).

significant difference of the putaminal T2 and iron values between the control group of this study and our previous results (7).

Globus Pallidus (see Table 2)

Iron concentration was found to be significantly higher in diseased subjects compared with normal controls ($P < .05$), although there was no significant difference between Huntington (191 μg/g, crude fraction) and Parkinson (181 μg/g, crude fraction) subjects at the 95% confidence level. Ferritin levels were significantly different between the three categories: Huntington (the highest), Parkinson, and normal (the lowest) ($P < .05$). There was no significant difference in T2 values between the three subject groups at the 95% confidence level. Again, no significant difference was demonstrated between the pallidal T2 and iron values in the control group of this study and our previous study (See Figs. 1-6).

Sources of Error

Ferritin Assay. Detailed review of our methodology suggested that the most likely source of error was inherent in the immunoassay for ferritin arising from lack of monoclonal antibody.

Antibodies against ferritin used in our experiments were raised by inoculating rabbits against purified human spleen ferritin. Antisera thus generated consists of a wide *mixture* of immunoglobulins each directed against different ferritin epitopes. An epitope (an antigenic determinant) represents an immunoglobulin binding site on the antigen, ie, ferritin. A protein the size of ferritin has many epitopes; hence, such antisera are polyclonal.

Human ferritin is composed of two classes of subunits: the heavy (H) and light (L) subunits totaling 24 subunits. The ratio of H to L subunits differs from one organ to another. Brain ferritin, therefore, will cross-react with spleen ferritin under anti-spleen ferritin antibodies, but it is not identical.

TABLE 2: Globus pallidus: T2, iron, and ferritin

	T2 (msec)	Iron, soluble (μg/g)	Iron, crude (μg/g)	Ferritin (μg/g)
Normal (n = 6)	76 ± 12	48 ± 15	134 ± 43	1.0 ± 0.2
Parkinson (n = 10)	67 ± 4	63 ± 12	179 ± 24	1.8 ± 1.1
Huntington (n = 6)	73 ± 7	80 ± 71	197 ± 34	3.3 ± 1.1

Note.—The soluble and crude iron levels are expressed in micrograms per wet gram of tissue (μg/g). The ferritin values are numerical ratio of the mean control (normal) values in putamen or globus pallidus. The T2 values are in milliseconds (msec).

Ferritin concentration was measured using immunoradiometric assay in which solid phase adhered primary antibodies and radioactive secondary antibodies. The ligands (ie, ferritin molecules) are bound to the primary antibodies and hence to the solid phase, whereas unbound ligands and nonligands are washed off. Secondary antibodies then bind the ligands, and radioactivity count is taken. Because the assay relies on the ligand binding activity of the primary and secondary antibody, a relatively small change in the counts is greatly amplified in the final ferritin concentration because of the nonlinear relationship between the radioactivity and ligand concentration.

As crude ferritin represents both ferritin and hemosiderin, and as no universally accepted assay for ferritin exists, this was not included in this paper.

Storage of Tissue. Our previous experiments (unpublished data) demonstrated relatively little (<10%) decrease in T2 with prolonged airtight freezing at temperatures ranging from −10°C to −60°C. We do not expect freezing to cause major alteration in iron distribution among the iron species (see Discussion). The protein shell components of the ferritin (and likely hemosiderin) are stable, antigenically and biochemically, under the above storage condition. Thus storage under −60°C was deemed to be less important.

Discussion

The presence of high concentrations of iron in tissue can cause T2 relaxation enhancement and thereby reduction in MR signal intensity. This effect is apparent on T2-weighted spin-echo MR images, and even more so on gradient recalled-echo MR images due to T2* effects. The mechanism for T2 relaxation enhancement on spin-echo images is that spins diffusing through iron-induced magnetic field perturbations undergo additional dephasing that cannot be compensated for by radiofrequency refocusing (15). Hence the signal is lower than it would be if the iron were not present. The effect is most evident at high field strength.

Gradient-echo sequences are more sensitive to the signal-reducing effects of iron because they do not utilize a refocusing pulse. Susceptibility differences and other intrinsic inhomogeneities induced by iron deposition cause additional spin dephasing which is not compensated for on the

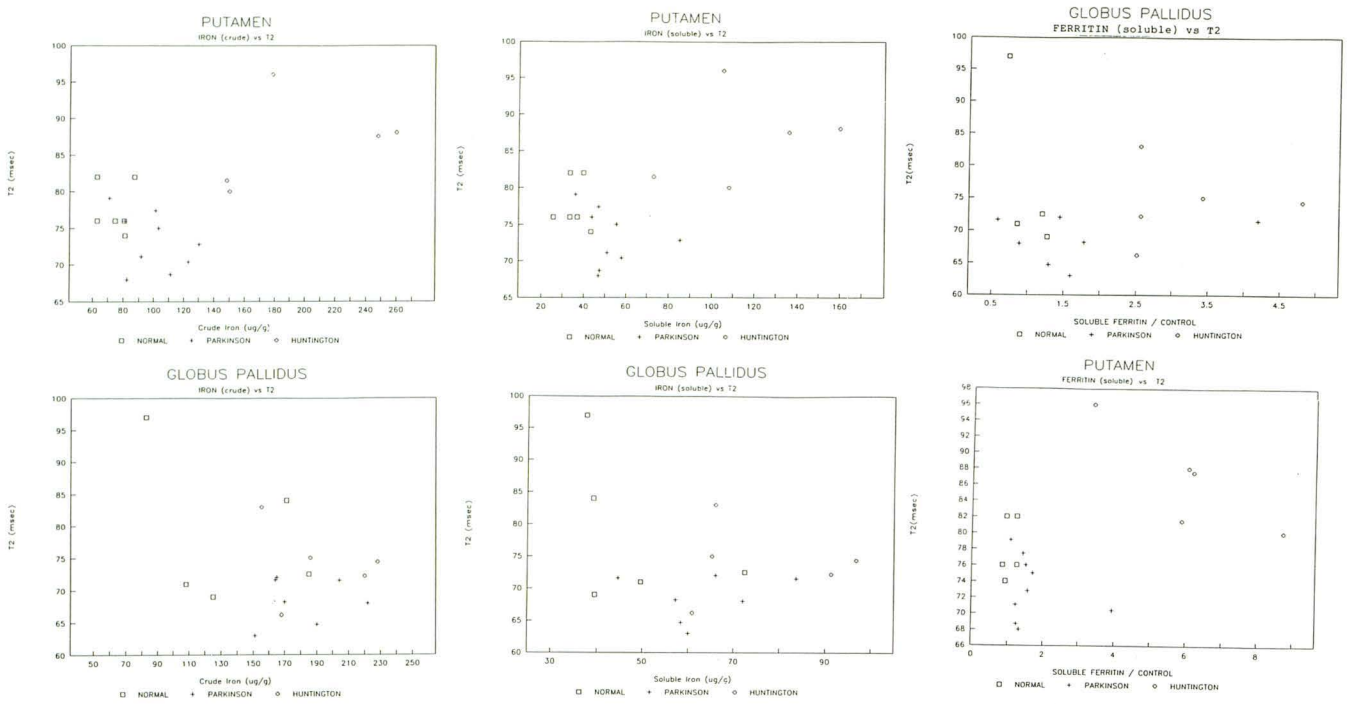

Fig. 1 to 6. T2 relaxation as a function of iron and ferritin concentrations. T2 is represented on the vertical axis (msec). The absolute value of iron concentration (μg/g) and relative value of ferritin (the mean of the control = 1.0) are represented on the horizontal axis. There is no relationship demonstrated between T2 and iron concentration, or between T2 and ferritin in any of these graphs.

gradient-echo sequences. T2* is the parameter that describes this spin dephasing on gradient-echo sequences; it is the parameter that governs image contrast on gradient-echo images. The term T2* is not applicable to spin-echo images.

It is important to recognize that while gradient-echo techniques are more sensitive to the presence of iron, it is spin-echo techniques that have been reported in the literature demonstrating abnormal areas of hypointensity in various diseases. Therefore, it is spin-echo methods that we have focused on. It is also important to note that any spin-echo method will rephase susceptibility effects and give a signal intensity weighted by T2 independent of the 180° pulse spacings provided the spins are static. Even for motionally dephased spins, the effect of 180° pulse spacing is small (15). Therefore, the fact that we used 2-msec spacings between 180° pulses in our relaxometer measurements cannot account for signal or T2 differences when compared with a whole body imager where T2-weighted spin-echo sequences typically utilize two echoes with 40 to 60 msec spacings.

Iron deposition in the brain is mainly in organic storage forms such as ferritin and hemosiderin. These accumulate under normal physiologic processes. Iron is also present in calcific deposits and in the atheroma of vessel walls. Only a very minute amount of free ionic iron exists intra- and extracellularly. Studies of iron distribution in the brain have demonstrated that there is a relatively higher concentration of iron in the basal ganglia, and that the degree of iron deposition increases with age and in certain diseases, most notably the extrapyramidal movement disorders (11, 14, 16–20). Furthermore, MR examinations of the brain in the elderly and in patients with extrapyramidal movement disorders (in particular Parkinson disease) often show hypointensity in the basal ganglia. This geographic association between MR hypointensity and localization of iron has led many investigators to conclude that iron is the factor primarily responsible for the reduced signal intensity observed on MR images, and conversely that hypointensity is indicative of iron deposition in diseased brain tissue (3, 21–30). The reasoning has been that because iron is present in these tissues and because iron can reduce signal intensity, iron must be the agent responsible for the reduced signal intensity that is observed. However, in an earlier investigation we were not able to demonstrate any quantitative correlation between iron concentration and T2 relaxation. Our investigation differed from others in that we utilized *quantitative* assays of iron (flame atomic

absorption spectrometry) and correlated the iron concentrations so obtained directly with imager and spectrometer-determined T2 values of the brain tissue (7).

The influence of iron on MR signal intensity has been quantitatively examined by others but mostly in noncerebral tissues. For example, it has been demonstrated that iron and ferritin levels correlate with reduction in signal intensity on T2-weighted images in the iron-overloaded liver and spleen. Gomori et al demonstrated a decrease in signal intensity with increasing iron concentration in the thalassemic spleen in vitro, and in the hemochromatotic liver in vivo and in vitro. The concentration of iron in Gomori's specimens ranged from 101 μg/g to 10,629 μg/g in the splenic tissue (1) and 3,000 μg/g to 9,000 μg/g in the hepatic tissue (6). Stark et al demonstrated similar results in livers of patients with hemochromatosis, and in an experimental rat model of liver iron overload. They reported concentrations ranging from 500 μg/g to 3,000 μg/g in the human sample and 1,200 μg/g to 4,500 μg/g in rats (2, 5).

Thulborn et al demonstrated significant reduction in signal intensity on T2-weighted images in a rat model of cerebral hemorrhage. Here, mean local iron concentrations of 800 μg/g over the control level of 200 to 300 μg/g were achieved in rat caudate nuclei through stereotactic injection of blood (4). Aoki et al have observed earliest T2-weighted signal reduction in globus pallidus in prepubertal patients and have attributed this to local iron accumulation (25). By noting the previously published local iron concentration (20) for this region, they have speculated 100 to 150 μg/g to be a critical level of iron for *detection* on MR images. However, no direct quantitative correlation was made to substantiate that the hypointensity is mediated by iron alone, or that increasing iron levels will correspondingly further reduce signal intensity. Brown et al postulated a critical level of detection at 50 μmol/g (2800 μg/g) (31), whereas Stark et al reported that 1200 μg/g would be required in liver tissue using MR imaging (2, 5). In the normal brain, the concentration range of iron is 20 to 200 μg/g depending on the brain region and age (11, 14, 17, 20), one to three orders of magnitude less than the "thresholds" found in these studies involving iron-overloaded spleen and liver, and murine cerebral hemorrhage models.

In a previous investigation of the relationship between tissue iron concentration and T2 relaxation, we reported that, in the brains of neurologically intact individuals, iron levels ranged from 15 μg/g to 134 μg/g in the basal ganglia (7). These concentrations are considerably below the thresholds other investigators reported as necessary to affect T2 in other tissues. In keeping with the threshold hypotheses, we found no consistent correlation between regional iron levels and spectrometer-derived T2 values. In an attempt to study the effect of higher iron concentration, we added ferric chloride and ferritin to *homogenized* brain tissue. We found that a relaxation rate change did occur but not until approximately 1,200 μg/g of ferritin or 350 μg/g of ferric chloride was present in the homogenate (7). We speculated that the tissue acted to bind iron and hence sequester iron from effective interaction with water, this would effectively reduce the relaxivity of the iron species until the binding sites were saturated. We suggest that for a given tissue there exists a critical concentration for the iron species. Above this level, the effect of iron on local water environment becomes the major determinant on T2-weighted signal intensity. If this were true, the sequestration of iron in native brain tissue could explain the lack of correlation between iron concentration and T2 values at the relatively low concentration of iron found in the normal brain.

The brains of patients with extrapyramidal movement disorders are good models in which increased concentrations of tissue iron can be studied with MR. Increased basal ganglia iron is well documented in these diseases, including Parkinson and Huntington diseases (11–14). Our assays show a clear increase in the iron levels in both Parkinson disease and Huntington disease. The relative distribution of iron may also be abnormal in these diseases. In the normal brain, the globus pallidus has more iron than the putamen. In Parkinson disease, the amount of iron in both these anatomic regions is increased as it is in Huntington disease, but in Huntington disease, the amount of iron in the putamen may equal or even exceed that in the globus pallidus (11).

There is a trend of T2 shortening with increasing iron concentration in Parkinson disease in both the putamen and globus pallidus as well as in the globus pallidus in Huntington disease, although only that of the putamen is statistically significant. This agrees with the theories of previous authors (21–27, 30). However, the reverse is observed in putamen samples from Huntington disease brains. In spite of clearly elevated iron

and ferritin levels, the T2 values are higher, not lower, than that of the normal brain. Clearly a factor other than iron must be the main determinant of signal intensity in the putamen in Huntington disease. Interestingly, the MR literature has described both an increase and decrease in the T2-weighted signal intensity of the basal ganglia in Huntington disease. Some have reported a signal reduction (24, 32), whereas Sax reported a prolongation of T2 values and attributed this higher signal intensity to an increase in gliotic tissue (33).

In a murine brain model, a significant (40% to 60%) reduction in regional T2 values has been demonstrated in the regions of low iron concentration such as gray matter and corpus callosum, as well as in the basal ganglia and substantia nigra, by increasing static field strength from 2.0 T to 7.0 T. With the exception of the substantia nigra, no clear iron-dependent T2 reduction was demonstrated at either field strength (34). Although the authors acknowledge the possible contribution of iron in relaxation enhancement in the iron rich regions of the brain, they speculate that the diffusion of the protons through local field perturbation independent of the regional iron concentration as the fundamental mechanism to explain this observation.

It is most likely that the signal intensity of the basal ganglia on T2-weighted MR images is determined by multiple factors, the concentration of iron being only one of these. The degree of contribution by the various forms of iron in tissue is not clearly known. We believe that, at the concentration range of storage iron encountered in the brains of normal subjects and in the brains of subjects with extrapyramidal movement disorders, the contribution by iron to T2-weighted signal intensity is not as great as previously hypothesized. The fact that iron deposition in tissue can cause a reduction in T2 relaxation is not disputed. However, at the concentrations of iron found in the brain of both normal subjects and those with Huntington and Parkinson disease, we have found no correlation between iron and T2. It may very well be that the experimental errors inherent in excising small anatomic structures, measuring T2, and assaying postmortem tissue may mask the correlation between iron concentration and T2 relaxation if one did exist. However, there is the obvious discrepancy of Huntington disease, where the highest amount of tissue iron was found in the putamen yet the signal intensity of the putamen was increased, not decreased. Clearly, there are other processes present in the putamen to account for the observed hyperintensity of the tissue and these processes dominate any reduction in signal intensity that the iron which is present in that tissue might cause. Therefore, at a minimum, the observation of *hyperintensity* in the basal ganglia *must not be attributed to reduced iron* concentration, and, conversely, this study suggests that the inference that *hypointensity in the basal ganglia implies increased iron accumulation cannot be quantitatively supported.*

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