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Paramagnetic Contribution of Serum Iron to the Spin-Spin Relaxation Rate ($1/T_2$) Measured by MRI

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Abstract. Spin-spin relaxation time T_2 values of serum with and without iron were measured by magnetic resonance imaging (MRI) to find the proton relaxivity of Fe(III) in serum. T_2 values in serum containing definite amounts of added iron were also measured before and after addition of ascorbic acid. The difference in the $1/T_2$ of serum with and without ascorbic acid was used for recalculation of the added iron values. Recalculated iron values confirm that the difference in healthy serum is caused by iron only. In addition, in order to find the paramagnetic contribution of serum iron, T_2 values of iron-deficient, healthy and iron-overloaded serum were measured before and after addition of ascorbic acid. The difference in the $1/T_2$ values was then applied to the calculation of the serum iron values. The consistency of iron values determined from the difference to those by autoanalyzer suggests that the differences in diseased serum also represent the paramagnetic contribution of serum iron. The data imply that serum iron content in healthy, iron-deficient and iron-overloaded serum may be assessed by MRI.

1 Introduction

It has been known that the presence of ferric iron in a solution markedly increases the spin-spin relaxation rate ($1/T_2$) of solvent water protons [1, 2]. The increase in relaxation rate caused by paramagnetic ions in a solution is called the paramagnetic contribution (PMC). The paramagnetic contribution can be defined as the difference between the observed relaxation rates of a solution in the presence and the absence of ions [3, 4]. The PMC per unit concentration of added ion defines the proton relaxivity of the ion. The proton relaxivity of ferric iron in a solution increases as the pH of the solution decreases [1, 5–7].

Nuclear magnetic resonance (NMR) studies on the relaxation rates of transferrin and serum have shown that ferric iron contributes to the spin-lattice relaxation rate of serum [1, 5, 6, 8, 9]. This contribution is barely detectable at physiological pH, but it becomes larger at lower pH [1, 5–7]. The magnitude of

the contribution was measured by both NMR and magnetic resonance imaging (MRI) [5, 6]. On the other hand, the spin-spin relaxation time (T_2) has also been measured in serum, but determinants of water proton T_2 in serum have not been studied yet. The assessment of the PMC of serum iron to $1/T_2$ in serum should therefore be of a scientific interest.

Adding ascorbic acid to serum reduces ferric iron to its ferrous form and removes the PMC of ferric iron to $1/T_1$, in serum [5, 6, 10, 11]. The loss of the PMC can cause a decrease in $1/T_2$. Hence measuring the $1/T_2$ rates of serum before and after addition of ascorbic acid should be utilized for determining the PMC of serum iron.

This work aims to study the PMC of serum iron to $1/T_2$ in serum. This was made at two stages. At the first stage it was confirmed that the difference in relaxation rates measured before and after addition of ascorbic acid to serum is caused by iron only. For this purpose, firstly proton relaxivities of various serum ions, such as ferric iron, ferrous iron and copper, were determined. Then definite amounts of iron added to serum were recalculated by using the difference in relaxation rates measured before and after addition of ascorbic acid. At the second stage the PMC in healthy, iron-deficient, and iron-overloaded serum were determined from the difference of T_2 values measured before and after addition of ascorbic acid. For comparative purposes, the PMC of each serum was utilized for determination of its iron content, which was also determined by an autoanalyzer.

2 Materials and Methods

2.1 Serum Samples

Several pooled sera were prepared from healthy volunteers, while three different groups of serum samples were prepared from patients with iron deficiency (some of them with protein deficiency), patients with iron overload, and also from healthy individuals. The pH of each sample was adjusted to 1.6 with a little amount of concentrated phosphoric acid. The samples were transferred into cylindrical glass tubes (1.3 cm in diameter and 10 cm height), which were placed in phantoms containing agar. The volume of each sample was 4 ml.

The pooled sera were used at the first stage to determine the proton relaxivities and to recalculate added iron, whereas the samples in the groups were used at the second stage to determine the PMC in healthy and diseased serum.

Except phantoms used for the relaxivities, all phantoms were imaged before and after addition of ascorbic acid. The position of each phantom and all the other experimental parameters were kept constant for both images.

2.2 Stock Solutions

Stock solutions of copper, ferrous and ferric iron were prepared by dissolving weighted quantities of $CuCl_2$, $Fe(NO_3)_3$ and $FeSO_4$ (Farmitalia Carlo Erba) in

deionized water. The stock solutions were used for determinations of the proton relaxivities and also used for addition of iron to serum. The final concentrations in some cases were prepared by making serial dilutions of a stock solution of each ion with distilled water.

2.3 Relaxation Measurements

T_2 relaxation times were measured with two MRI devices operating at field strengths of 1 and 2.38 T. Higher-field MRI is being used for research, while the other is used for routine.

Relaxation measurements at 2.38 T were carried out on a Bruker Biospect MRI spectrometer with standard birdcage coil (inner diameter of 195 mm). A Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence was used to obtain images with repetition time (TR) of 5000 ms and echo delays were changed from 22.5 to 630 ms. The field of view (FOV) and slice thickness were 140 by 140 mm and 10 mm, respectively. With a 10 mm diameter circular region of interest within each tube, T_2 values were obtained from magnetization decay curves versus echo time (TE).

Relaxation measurements at 1 T were carried out with a Siemens magnetom SP 10 MR scanner. The CPMG pulse sequence with multi echoes was used. Echo delays were changed from 20 to 160 ms. TR, FOV and slice thickness were 5000 ms, 150 by 150 mm and 10 mm, respectively. In this case, T_2 was obtained from T_2 maps.

2.4 Determination of Proton Relaxivity (R)

The proton relaxivity of Fe(III) in serum was found in two different ways: (1) 5 μg of Fe(III) per ml was added to half of samples in a phantom with a pooled serum, in a successive order of serum with and without iron (Fig. 1a). T_2 values in samples were then determined after image construction. The proton relaxivity was calculated from the following equation [1, 2]

$$R = (1/T_{2i} - 1/T_2)/C, \tag{1}$$

where $1/T_{2i}$ and $1/T_2$ are the mean relaxation rates of samples with and without iron, respectively, and C is the concentration of added iron in mM. The difference of relaxation rates $1/T_{2i} - 1/T_2$ shows the PMC of added iron in reciprocal seconds. (2) Three sets of tubes in a phantom prepared from a pooled serum were allocated to determine the proton relaxivities of Fe(III), Fe(II) and Cu(II) in serum (Fig. 1b). Each set, containing five tubes, was used for one of these ions. The concentration of iron was increased from 0.036 to 1.44 mM, while the concentration of Cu(II) was altered from 0.032 to 1.28 mM. T_2 values of each set were determined after the phantom was imaged. The relaxation rates were plotted versus concentrations of Fe(III), Fe(II) and Cu(II). The slope of each relation was taken as proton relaxivity [12].

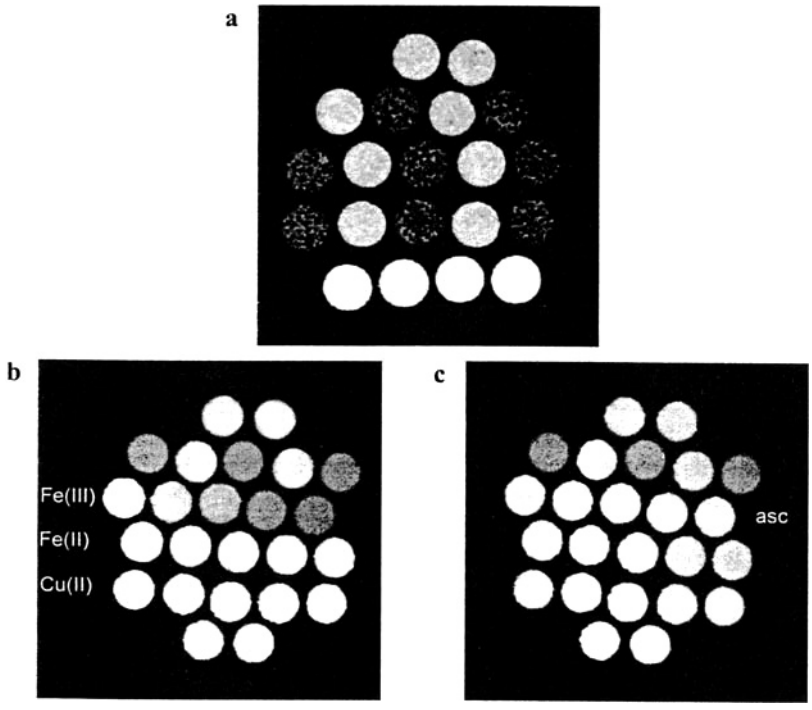

Fig. 1. **a** SE image of a pooled serum with and without 5 μg of ferric iron per ml. **b** T_2 map of a phantom containing a pooled serum with iron and copper. (From top to bottom: the second row shows serum samples with and without 5 μg of iron per ml, whereas third, fourth and fifth rows show the sets containing Fe(III), Fe(II) and Cu(II), respectively.) **c** T_2 map of the same phantom after addition of ascorbic acid to the set with Fe(III). **a** 2.38 T, **b** and **c** 1 T.

To determine whether Fe(III) is completely reduced to Fe(II), ascorbic acid was added to tubes in the set designed for the proton relaxivity of Fe(III), and then the phantom was imaged once more (Fig. 1c). After measuring T_2 values of the tubes, the proton relaxivity of reduced iron was determined from the slope of the relation between the $1/T_2$ and the concentration of iron. The relaxivities of Fe(II), reduced iron and Cu(II) were used for comparison with that of Fe(III).

2.5 Recalculation of Added Iron

In order to see whether the relaxation rate difference in serum is only due to serum iron, certain amounts of iron added to serum were recalculated by using the difference. For this purpose, the iron content in a pooled serum was measured as 110 μg/dl by an autoanalyzer. With this serum three types of samples differing largely in iron concentration were prepared (Fig. 2a). The iron content of each sample in one type was reduced to 37 μg/dl by diluting the pooled serum to 2/3, while the iron content of each sample in another type was increased

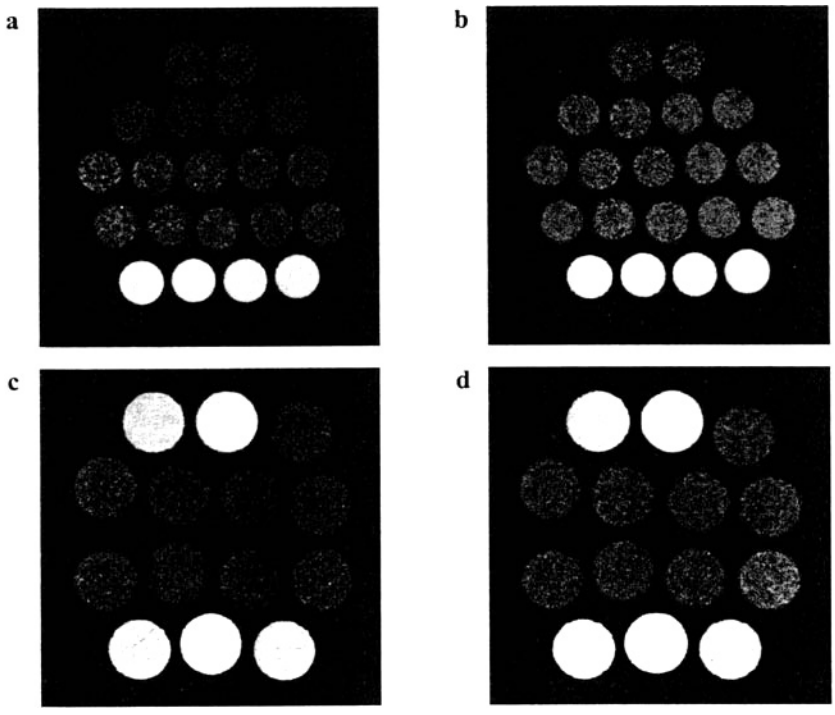

Fig. 2. SE images of a phantom containing a diluted pooled serum (bottom row), pooled serum (brighter medium rows) and pooled serum with added iron (darker top rows), before (**a**) and after (**b**) addition of ascorbic acid. **c** A phantom containing pooled serum with various concentrations of added iron, **d** the image of the same phantom after addition ascorbic acid. Iron concentration in some successive tubes (darker ones) increased in steps of 12.5 μg/ml. Diluted serum (brighter three) and water samples (brightest two) were also included. The data were taken at 2.38 T.

to 330 μg/dl by adding Fe(III) to the pooled serum. The third type was the pooled serum. Furthermore, to prepare samples comparable with healthy, iron-deficient and iron-overloaded serum, increasing amounts of iron over a broad concentration range were added to another pooled serum (Fig. 2b). In this case the iron content of pooled serum, determined by autoanalyzer, was found to be 133 μg/dl. The iron concentrations of eight consecutive tubes were increased from 133 to 220 μg/dl in steps of 12.5 μg/dl (Fig. 2c). A healthy serum, the diluted serum and distilled water were also included.

The relaxation rates of the phantoms were measured before (Fig. 2a, c) and after addition of ascorbic acid (Fig. 2b, d). The difference of the relaxation rates measured before ($1/T_{2b}$) and after ($1/T_{2a}$) was considered as the PMC of iron to serum. Iron content in serum was then calculated from the following formula [1, 5, 6]

$$C = \frac{1/T_{2b} - 1/T_{2a}}{R} \cdot 55.847 \cdot 100\,, \tag{2}$$

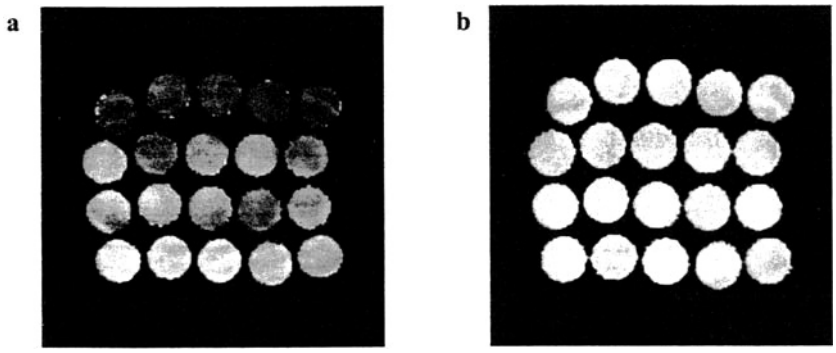

Fig. 3. T_2 maps of iron-deficient, healthy and iron-overloaded serum samples constructed before (**a**) and after (**b**) addition of ascorbic acid. The top row shows iron-overloaded sera, whereas the two medium rows and the bottom row show healthy and iron-deficient sera, respectively. The data were taken at 1 T.

where 55.847 is molecular weight of iron. This equation was derived from the definition of the proton relaxivity (Eq. (1)) by converting the concentration unit from mM to μg/dl.

2.6 Determination of the Paramagnetic Contribution of Iron in Healthy and Diseased Serum

The relaxation times of healthy, iron-overloaded, iron-deficient samples were measured before and after addition of ascorbic acid (Fig. 3a, b). As in the case of samples with added iron, the difference of the relaxation rates measured before and after addition of ascorbic acid was considered as the PMC of iron to $1/T_2$ in serum. To confirm this point further, the iron content in each serum was then calculated from Eq. (2) by using the difference. For comparative purposes, iron contents of the same samples were also determined by an autoanalyzer (Toshiba, Abbott-Aeroset).

Experiments on the relaxivities and recalculation of iron added and also on the differences in the healthy and diseased serum were repeated several times with many different phantoms.

3 Results and Discussion

The relaxivities of Fe(III) in serum and diluted serum containing 5 μg of added iron per dl are shown in Table 1, whereas the relaxation rates in the sets versus several concentrations of Fe(III), Fe(II), reduced iron and Cu(II) are given in Fig. 4. It is seen that the relaxation rates are linearly proportional to the concentrations. The slope of each line represents the relaxivity of the corresponding ion. The proton relaxivity of Fe(III) at 2.38 T was 13.7 $mM^{-1}s^{-1}$, while the relaxivities

Table 1. Proton relaxivities of Fe(III) in serum. The differences in $1/T_2$ are obtained from the mean values in pooled serum without ($1/T_2$) and with iron ($1/T_{2i}$).

Serum	Fields (T)	Nr. of experiments	$1/T_2$ (s^{-1})	$1/T_{2i}$ (s^{-1})	Difference in $1/T_2$ (s^{-1})	R ($mM^{-1}s^{-1}$)
Healthy	1	21	3.655±0.05	4.789±0.05	1.134	12.67
Healthy	2.38	27	2.618±0.03	3.847±0.03	1.229	13.70
Diluted	2.38	13	1.090±0.03	2.307±0.03	1.217	13.60

of Fe(III), Fe(II), reduced iron and Cu(II) at 1 T were 12.46, 0.190, 0.196 and 0.227 $mM^{-1}s^{-1}$, respectively. The dilution of serum did not affect the relaxivity significantly.

The differences in pooled serum, diluted pooled serum and pooled serum with added iron together with their iron contents are given in Table 2, whereas the differences in healthy and diseased sera and also their iron contents are given in Table 3. The difference in diseased serum ranged from 0.078 to 0.551 s^{-1} at 1 T, while it ranged from 0.087 to 0.538 s^{-1} at 2.38 T. Furthermore, the plot of calculated iron versus added iron is given in Fig. 5 for the samples containing added iron over a broad concentration range.

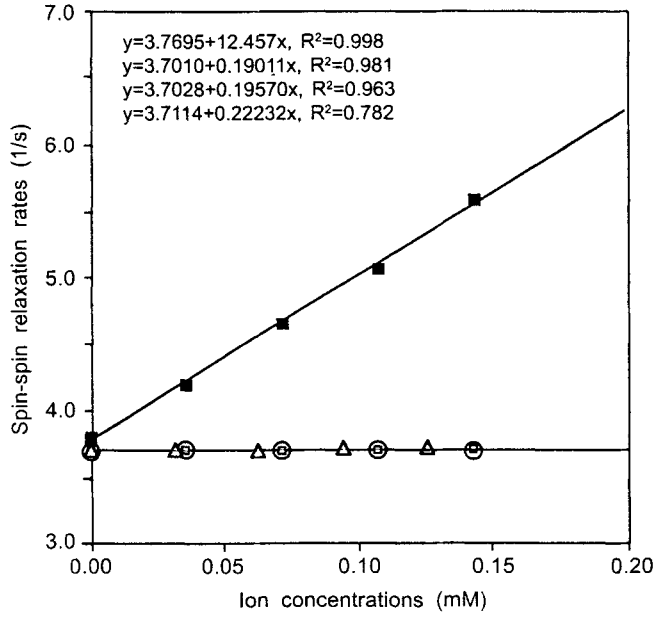

Fig. 4. Spin-spin relaxation rates in serum versus various concentrations of ferric iron (solid squares), ferrous iron (open circles), reduced iron (open squares) and copper (open triangles). The order of the equations is the same as that of symbols. The data were taken at 1 T.

Table 2. Relaxation rates in diluted pooled serum, normal pooled serum and pooled serum with added iron. The differences in $1/T_2$ and iron values determined by MRI and an autoanalyzer are also given. T_{2b} and T_{2a} denote the relaxation times measured before and after addition of ascorbic acid, respectively. The results were taken at 2.38 T.

Serum	Nr. of experiments	$1/T_{2b}$ (s^{-1})	$1/T_{2a}$ (s^{-1})	Difference in $1/T_2$ (s^{-1})	Fe^{MRI} (μg/dl)	Fe^{auto} (μg/dl)
Diluted	8	1.086±0.03	0.966±0.03	0.120±0.010	49±4	37
Healthy	20	2.622±0.03	2.368±0.03	0.255±0.017	104±8	110
With iron	12	3.172±0.04	2.374±0.03	0.798±0.025	325±10	330

In the present study, the difference of relaxation rates measured before and after addition of ascorbic acid was attributed only to the PMC of serum iron. This is based on two assumptions. (1) Ascorbic acid cancels out only the PMC of iron and leaves the rest unaffected. (2) Fe(III) is completely reduced by ascorbic acid. The validities of these assumptions are discussed below.

3.1 Calculation of Added Iron by the Difference in the $1/T_2$

Serum consists of water, various proteins and ions. The main paramagnetic ions in serum are Fe(III) and Cu(II). Water, total protein (TP), serum iron are known to contribute to the relaxation rate of serum [1, 5, 6, 8, 9, 13]. This can be written as follows:

$$1/T_{2b} = 1/T_{2w} + 1/T_{2tp} + 1/T_{2si}, \tag{3}$$

where $1/T_{2b}$ is the relaxation rate of serum before adding ascorbic acid, and $1/T_{2w}$, $1/T_{2tp}$ and $1/T_{2si}$, are the contributions of water, TP and serum iron, respectively. The contribution of serum iron increases as pH decreases [1, 5–7].

Table 3. Relaxation rates in iron-deficient, healthy and iron-overloaded serum samples without and with ascorbic acid. The differences in $1/T_2$ and iron values determined by MRI and an auto-analyzer are also given.

Serum	Fields (T)	Nr. of experiments	$1/T_{2b}$ (s^{-1})	$1/T_{2a}$ (s^{-1})	Difference in $1/T_2$ (s^{-1})	Fe^{MRI} (μg/dl)	Fe^{auto} (μg/dl)
Iron-	1	19	3.441±0.361	3.363±0.362	0.078±0.050	34±22	37±15
deficient	2.38	10	2.413±0.212	2.326±0.205	0.087±0.021	36±9	40±9
Healthy	1	30	3.662±0.208	3.422±0.192	0.240±0.051	106±23	110±34
	2.38	20	2.631±0.190	2.361±0.129	0.268±0.066	109±27	108±36
Iron-	1	19	3.978±0.118	3.427±0.112	0.551±0.101	243±45	230±42
overloaded	2.38	10	2.903±0.133	2.365±0.102	0.538±0.081	219±33	228±38

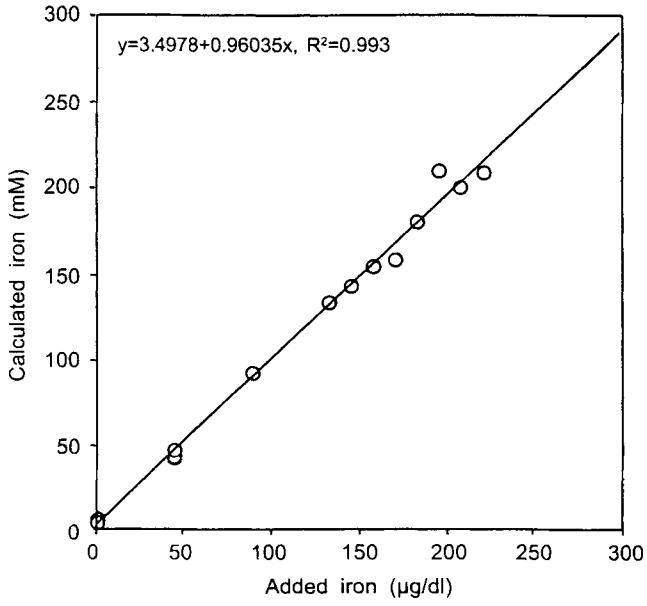

Fig. 5. Calculated iron versus iron added to serum over a broad concentration range. The data was taken at 2.38 T.

If ascorbic acid affects only the PMC, the first two terms on the right-hand of Eq. (3) remain as before and the relaxation rate of samples with ascorbic acid ($1/T_{2a}$) is written as

$$1/T_{2a} = 1/T_{2w} + 1/T_{2tp}. \tag{4}$$

Then the difference of Eqs. (3) and (4), $1/T_{2b} - 1/T_{2a}$, is equal to the PMC of serum iron $1/T_{2si}$.

As it is seen in Table 2, the mean difference of the relaxation rates in the pooled serum decreases with the dilution of the sample, but increases with the addition of iron. According to the first assumption, the changes in the difference are caused only by the changes in iron concentration. This was confirmed by calculation of definite iron contents in samples by using the difference. It is seen that calculated iron values are consistent with added iron. This shows that the difference is caused only by iron. It also implies that Fe(III) is effectively reduced to Fe(II) by ascorbic acid.

The assumption was further confirmed by the slope of calculated iron versus the iron added over a broad concentration range. The value of slope (0.96) in Fig. 5 means that increasing amounts of iron added to serum can be calculated by using the difference. This shows that MRI cannot only differentiate large concentration changes but also it can discern small changes in serum iron.

3.2 Reduction of Fe(III) to Fe(II) by Ascorbic Acid

Ascorbic acid is known to reduce Fe(III) to Fe(II) and it is routinely used for the determination of serum iron by chemical methods [10, 11]. In those methods, the determination of iron is based on its dissociation from transferrin in acid medium and the reduction to its ferrous state using ascorbic acid. Although the results in Table 2 and Fig. 5 imply an effective reduction of serum iron, the second assumption can be further studied by comparison of the relaxivity of ferrous iron to that of iron reduced. As shown in Fig. 4, after addition of ascorbic acid to the set with Fe(III), the proton relaxivity of added iron (0.196 $mM^{-1}s^{-1}$) becomes similar to that of ferrous iron (190 $mM^{-1}s^{-1}$). This indicates a complete reduction of Fe(III) to Fe(II) by ascorbic acid.

3.3 PMC in Healthy, Iron-Overloaded and Iron-Deficient Serum

Serum iron varies greatly from sample to sample. For example, in a healthy population serum iron concentration ranges from 70 to 180 μg/dl [14, 15]. Values lower than the lower limit are considered as iron deficiency, and higher than the upper limit are described as iron overload.

As it is seen in Table 3, the difference in the groups increases as the groups are arranged from iron-deficient to iron-overloaded. The calculation of the added iron by MRI (the first stage) shows that the difference in healthy serum is due to serum iron, and also it strongly implies that the differences in iron-deficient and iron-overloaded serum represent the PMC of serum iron to $1/T_2$. To confirm this, the differences were applied to the calculation of serum iron in each sample. Iron contents calculated by MRI were found to be consistent with those determined by an autoanalyzer and with those given in the literature [16, 17]. The data are also compatible with those obtained by NMR and MRI T_1 measurements [5, 6]. This indicates that the differences in diseased serum also represent the PMC of serum iron.

3.4 Paramagnetic Contribution of Other Ions to the Relaxation Rate in Serum

The other ions contributing to $1/T_1$ in serum are Cu(II) and reduced iron. The mean concentration of Cu(II) in serum was 0.021 mM, while the mean concentration of reduced iron was 0.02 mM. Replacing the relaxivities and the concentrations of these ions in Eq. (1) gives the paramagnetic contributions of Cu(II) and Fe(II) as 0.005 s^{-1} and 0.004 s^{-1}, respectively. Comparison of these values with the differences in Table 2 indicates that the contributions of Fe(II) and Cu(II) can be neglected.

3.5 Comparison of the Results Obtained with Different Machines

As a result of molecular motion, the dipolar interaction between the nucleus and the electron will fluctuate in time and will therefore give rise to relaxation. The

relationship most widely used to describe the relaxation of a nucleus by an unpaired electron is given by the Solomon-Bloembergen equations, which include the resonance frequency of the nucleus [18]. For this reason, relaxation by paramagnetic centers is generally believed to be frequency-dependent.

It is seen from Table 1 that the proton relaxivity of Fe(III) at 1 T is smaller than that at 2.38 T. In order to study the dependence of $1/T_2$ on frequency further, T_2 values in some of samples measured at 2.38 T were repeated at 300 MHz on a Bruker CXP 300 NMR spectrometer. While the PMC and the relaxivity of Fe(III) in healthy serum at 2.38 T (100 MHz) was 0.255 s^{-1} and 13.7 $mM^{-1}s^{-1}$, respectively, they were found to be 0.459 s^{-1} and 19.47 $mM^{-1}s^{-1}$ at 300 MHz. Iron contents of these samples determined from the difference were consistent with those in Table 3. This preliminary data is not shown. Although the difference and the relaxivity vary with frequency, division of the difference to the relaxivity gives similar results for serum iron measured by different machines.

3.6 Usefulness of Assessment of the PMC by MRI

Despite the presence of many conventional methods, studies on new methods for the determination of serum iron are being carried out [19–25], and the search for blood pool contrast agents with iron oxides for MRI is still of a great interest [26–29]. This may bring a new interest in studies on a material chelating with transferrin iron to determine only serum iron in vivo. In fact, 4,5-dihydroxybenzene-1,3 disulfonic acid (TIRON) was suggested for in vitro evaluation of total Fe(III) content in human serum transferrin [30]. The work was based on the relaxivity of the TIRON-Fe(III) complex in solution. Thus the data on the paramagnetic contribution of iron to serum and the relaxivity of Fe(III) in serum may be useful for studies on a new iron determination method.

4 Conclusions

The current study suggests that the difference of $1/T_2$ in serum measured before and after addition of ascorbic acid represents the PMC of serum iron, and also the data imply that serum iron content in healthy, iron-deficient and iron-overloaded serum may be assessed by MRI T_2 measurements.

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