Effects of Cell Membrane Disruption on the Relaxation Rates of Blood and Clot with Various Methemoglobin Concentrations

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The magnetic resonance imaging (MRI) characteristics of hemorrhage and clotted blood change with age. The effects of methemoglobin and cell membrane lysis, factors which in part may underlie this evolution of imaging characteristics, were studied using clotted and heparinized dog blood at various methemoglobin concentrations. Cell lysis did not alter the longitudinal relaxation rate ($1/T_1$) in clotted or unclotted samples. Membrane lysis altered significantly the transverse relaxation rate ($1/T_2$) in both clotted and unclotted samples. Lysed samples of oxygenated blood at 0% methemoglobin had significantly higher T_2 values than intact samples. At 0% methemoglobin, clotted samples had slightly but significantly shorter relaxation times than unclotted samples. Within the samples studied, large changes in the state of oxygenation and methemoglobin content were observed in less than 24 h. Such changes necessitate frequent monitoring of these parameters if serial studies are to be done.

Key words: magnetic resonance; methemoglobin; blood; clot; membrane lysis

MAGNETIC RESONANCE IMAGING (MRI) characteristics of hemorrhage[1–9] and clotted blood[10–12] change with age. The precise mechanisms involved in this evolution of imaging characteristics remain unclear and somewhat controversial, although methemoglobin formation, cell lysis, and hematocrit changes[13] are thought to be involved.

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The objective of the current study was to examine the effects of cell membrane disruption on the longitudinal and transverse relaxation rates ($1/T_1$ and $1/T_2$) of blood and clot at various methemoglobin concentrations.

Materials and Methods

Venous blood was obtained from six mongrel dogs (18 to 20 kg body weight) and heparinized (80 U/mL). The blood was oxygenated by swirling in a beaker and divided into three 60 mL aliquots. The hemoglobin in one aliquot was converted to 100% methemoglobin by adding 1 mg/mL of sodium nitrite ($NaNO_2$). Actual measured methemoglobin content ranged from 94.3% to 99.8%; the measured values are depicted in the data plots. For simplicity, this group subsequently will be referred to as >94% methemoglobin. Intermediate methemoglobin concentrations were formed in the second aliquot by adding 0.1 to 0.2 mg/mL $NaNO_2$. The third aliquot served as a control, and was allowed to maintain its native methemoglobin concentration without addition of $NaNO_2$. Each of these aliquots was divided in half, and the cell membranes in one part were disrupted by ultrasonication at 3°C. Cell count by laser hematology (Ortho Diagnostic Systems ELT-8/ds, Westwood MA) confirmed >99.8% lysis of erythrocytes in the sonicated samples.

Preparation of Clotted Samples

Two and one-half mL of each of the sonicated and unsonicated preparations were clotted in syringes by adding 100 U of thrombin (suspended in 200 μL normal saline). Clotted samples were extruded from the syringes 1 hour after addition of thrombin, transferred to plastic tubes, and suspended in 1.5 mL of normal saline.

All 12 (six clotted, 6 unclotted) preparations were scanned with 4 hours of sonification on a Signa 1.5 Tesla imager (GE Medical, Waukesha, WI) using a multi-slice technique. Slice thickness was 3 mm with a 3 mm gap; pixel size was 1.25 mm × 0.62 mm. Gd-DTPA standards were imaged with each group of samples. Relaxation times were calculated from a 16 mm^2 region of interest (21 pixels) using standard Signa software.[14] For each preparation, T_2 values were calculated from a spin echo (TR,TE) 2000,30/60 sequence; T_1 values were calculated from spin echo sequences using eight TRs ranging from 300 to 5000 ms, with a TE of 30 ms. Figure 1 summarizes this sample preparation. This method of sample preparation will be referred to as Method A.

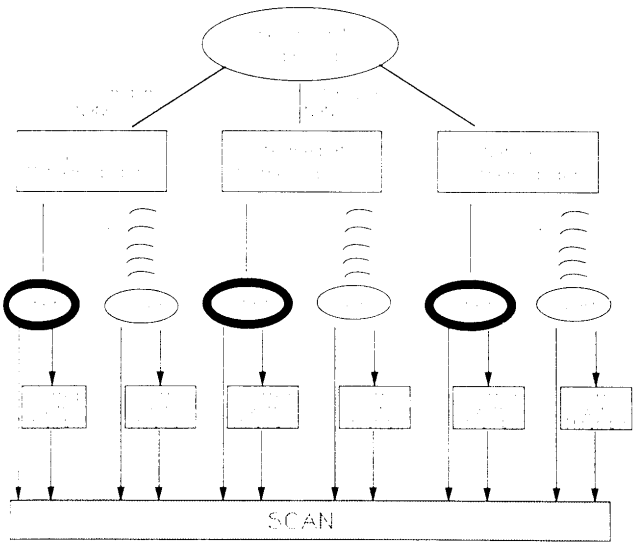

Fig. 1. Summary of experimental Method A.

Portions of all unclotted samples were analyzed spectrophotometrically for oxygen saturation and methemoglobin content using a blood gas analyzer (CO-Oximeter 282, Instrumentation Laboratory, Lexington, MA). Tubes were agitated before spectrophotometric sampling to avoid artifacts caused by cell settling. Spectrophotometric analyses were done concurrently with imaging so that measurements reflect sample contents at the time of imaging. Sample oxygenation was such that less than 5% of the total hemoglobin was in the deoxygenated form. Among the six dogs, hematocrit (mean ± SD was 43.8 ± 1.9%, and hemoglobin was 17.0 ± 1.2 g/dL.

Comparison of Clotted and Unclotted Samples

Direct comparison of the clotted and unclotted samples reported previously was not possible because of different preparation and handling. A second experimental method was devised to make such a comparison valid. This method was repeated six times.

For each of the three methemoglobin concentrations (>94%, intermediate, and 0%), 3.2 mL samples of intact (unsonicated) blood were clotted directly in the imaging tubes using 100 U thrombin suspended in 270 μL normal saline. Unclotted 3.2 mL samples of each methemoglobin preparation were diluted with 270 μL normal saline and placed in the imaging tubes. Imaging was done within 2 hours of the addition of thrombin. Hemoglobin and methemoglobin content were determined as stated previously. Imaging and blood gas analysis were repeated for these samples 24 hours after the first imaging. This method of sample preparation will be referred to as Method B.

Data Analysis

All comparisons between clotted and unclotted samples are based on data collected using Method B. All comparisons of lysed and unlysed samples are based on data collected using Method A reported previously.

Data were fitted using simple linear regression (Cricket Software, Malverne, PA). Student's t test was used to compare samples (SAS Institute, Cary, NC).

Results

There was no significant difference in the longitudinal relaxation rate ($1/T_1$) between sonicated and unsonicated blood samples (Fig. 2). The longitudinal relaxation rate increased in all samples as the methemoglobin concentration (expressed as percent of total heme protein) was increased. A similar increase in longitudinal relaxation rate was seen in clotted samples prepared using Method A. In these samples, cell lysis did not alter the relaxation rate (Fig. 3).

Considerable variation in the transverse relaxation rate ($1/T_2$) of sonicated samples was seen between different runs of the experiment. This may have been caused by variable inactivation of methemoglobin reductase during sonification or variation in the time between sonification and scan (2 to 4 h). Factors that might potentially inactivate methemoglobin reductase include damage to molecular subunits during sonification, and denaturation resulting from the heat of sonification. There was no variation (SD < 2%) in the T_2 of Gd-DTPA standards imaged with each group of samples. Therefore, the variability is intrinsic to the samples themselves and does not result from variation in the imaging process. Significant differences were apparent between intact and sonicated samples despite this variation.

Whereas the transverse relaxation rates of unsonicated samples (both clotted and unclotted) increased as the methemoglobin percentage increased, the transverse relaxation rates of sonicated samples were much less dependent on methemoglobin content (Figs. 4 and 5). The transverse relaxation rates of the sonicated samples was almost constant as percent methemoglobin increased.

In oxygenated samples at native methemoglobin concentrations (0% to 1.8%), membrane disruption resulted in a significantly ($P < .03$) higher T_2 in lysed samples (110.7 ±

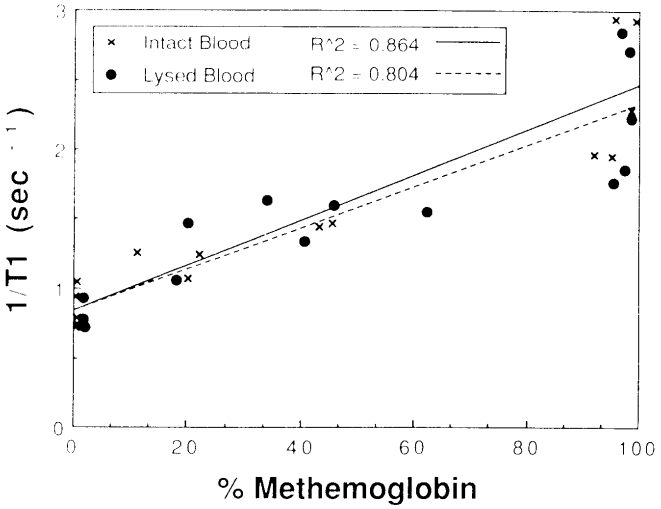

Fig. 2. Comparison of longitudinal relaxation rates in lysed and intact blood samples.

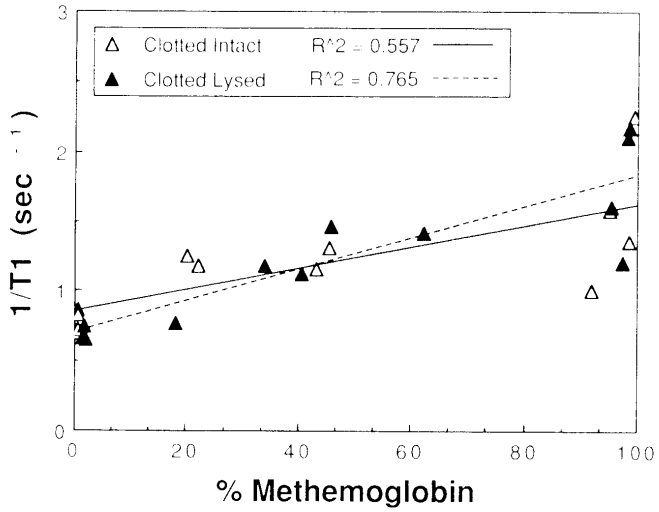

Fig. 3. Comparison of longitudinal relaxation rates in clotted lysed and intact samples.

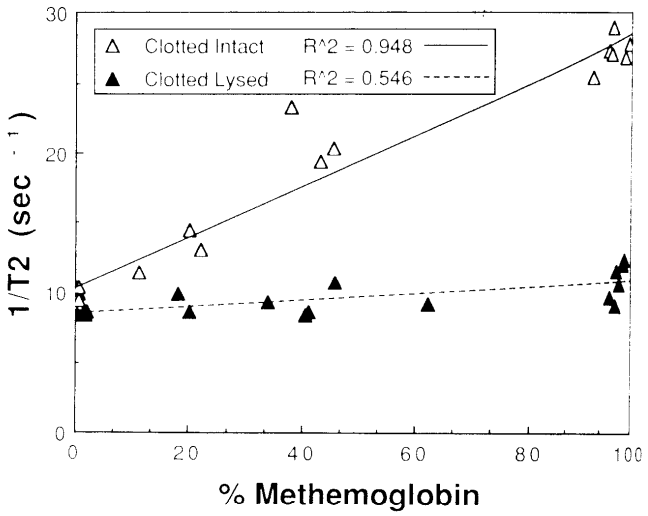

Fig. 5. Comparison of transverse relaxation rates in clotted lysed and intact samples.

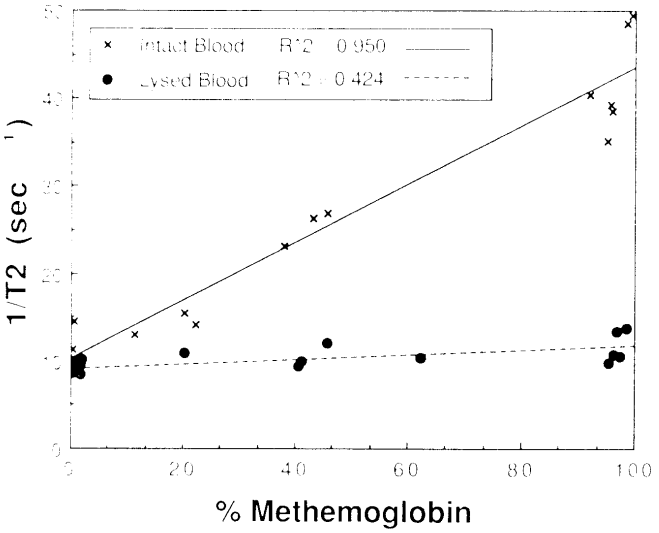

Fig. 4. Comparison of transverse relaxation rates in lysed and intact blood samples.

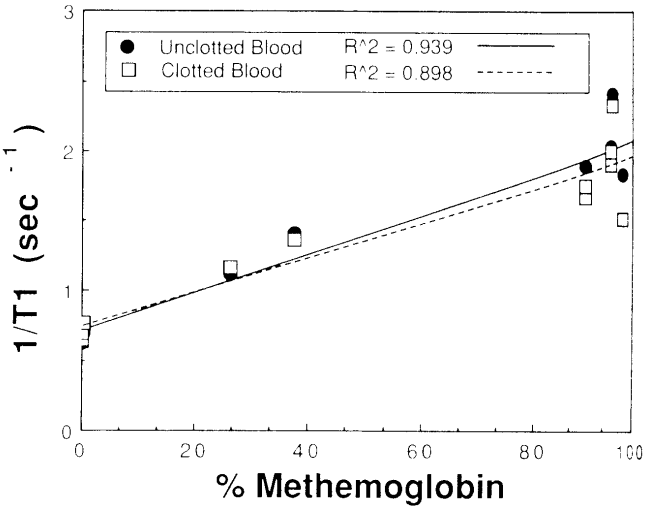

Fig. 6. Comparison of longitudinal relaxation rates in clotted and unclotted samples prepared using Method B.

6.3) than that found for intact samples (97.6 ± 5.9). No significant difference was found for T_1 (1249 ± 152 and 1208 ± 193, respectively).

Comparison of clotted and unclotted samples of intact blood (Method B) showed similar increases in relaxation rates as methemoglobin increased (Figs. 6 and 7). At native methemoglobin concentrations, clotted samples had slightly, but significantly ($P < .05$), shorter relaxation times than unclotted samples. At >94% methemoglobin, however, no statistically significant differences were found (Table 1).

The authors of the current study wanted to assess the effects of clot retraction by measuring T_1 and T_2 in 24-hour-old clots. However, measurements at 0 and 24 hours of methemoglobin content and hemoglobin saturation showed complex changes in the samples that precluded simple analysis based on clot retraction (Table 2).

Discussion

A neutral ion atom has 26 electrons. Of these, 18 reside in closed shells ($1s^2 2s^2 2p^6 3s^2 3p^6$); the remaining eight electrons have the oribital configuration ($3d^6 4s^2$). Ferrous iron (Fe^{2+}) has an electron orbital configuration outside the closed shells of ($3d^6$), in ferric iron (Fe^{3+}) the configuration is ($3d^5$). The 3d shell consists of five orbitals.[15] In the absence of a ligand, all five d orbitals of ferrous iron are energetically equivalent. In the presence of a ligand however, electrons of the donor atom of a ligand exert repulsion on the d orbital electrons. Two of the d orbitals face the ligand directly and become high-energy orbitals.[16] The

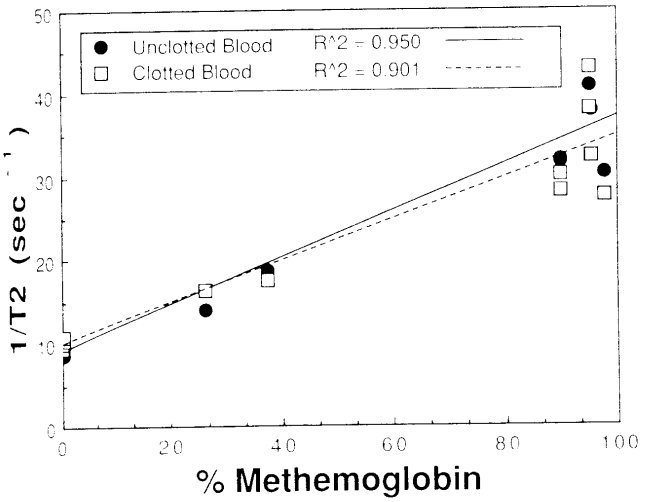

Fig. 7. Comparison of transverse relaxation rates in clotted and unclotted samples prepared using Method B.

TABLE 1. Relaxation Times of Clotted and Unclotted Samples

	T_2		T_1	
Sample	Unclotted	Clotted	Unclotted	Clotted
0% MetHb	106.0 ± 5.0	99.6 ± 4.8	1483 ± 91	1421 ± 109
100% MetHb	28.6 ± 3.8	30.9 ± 5.2	516 ± 58	546 ± 80

TABLE 2. Changes Within Samples Over 24 Hours

	MetHb	O_2Hb	Hb	COHb
100% MetHb				
0 h	95.4	4.8	0.0	0.3
24 h	72.3	13.6	14.1	1.1
Intermediate				
0 h	37.5	62.1	1.1	0.0
24 h	28.8	20.9	48.8	1.1

MetHb, O_2Hb, and COHb measured directly, the percentage deoxyhemoglobin (Hb) was calculated based on these values. Percentages do not always total 100%; this represents errors within the measurement system.

high-energy orbitals are termed e_g orbitals, the lower energy orbitals are t_{2g} orbitals. If the ligand exerts a weak repulsive force, the energy difference (Δ) between e_g and t_{2g} orbitals is small. A ligand with a strong repulsive force results in a large Δ.

In oxyhemoglobin, the ligand is oxygen that exerts a strong ligand field effect. Accordingly, the energy difference between e_g and t_{2g} orbitals is large. Δ is so large that the e_g orbitals are energetically unaccessable to the electrons. All six electrons remain in the t_{2g} orbitals where they are paired. Thus, oxyhemoglobin has no unpaired electrons. In deoxyhemoglobin, only a weak ligand field is exerted and Δ is small. Electrons can enter the e_g orbitals, and deoxyhemoglobin has four unpaired electrons. The energy difference between oribtals is small in methemoglobin, and electrons can transfer up into the e_g orbitals. During oxidation from deoxyhemoglobin to methemoglobin, an electron is lost and methemoglobin has five electrons in its 3d shell. Methemoglobin thus has five unpaired electrons.

The unpaired electrons of deoxyhemoglobin are protected from the surrounding solvent water within a nonpolar pocket of the protein. A conformational change occurs on conversion to methemoglobin such that its five unpaired electrons are freer to interact with the solvent water. The additional unpaired electron coupled with the accessability of those electrons render methemoglobin a more effective paramagnetic agent than deoxyhemoglobin. The mechanisms of paramagnetic solvent proton relaxation by methemoglobin are complex,[17–19] but it is clear that both inner sphere (ligand-exchange at the bound water molecule) and outer sphere (diffusional) effects contribute significantly to relaxation.[19]

The increase in longitudinal relaxation rate observed with increasing methemoglobin is due to a direct paramagnetic effect resulting from proton-electron dipole-dipole interaction. The T_1 relaxation enhancement was found to be roughly equivalent in intact and lysed blood samples. This is consistent with the findings of Thulborn,[20] but contrasts with the findings of Gomori et al.[21] Working at 0.94 T, Gomori et al found erythrocyte lysis to reduce T_1 by approximately 50% in samples with 100% MetHb. They attributed this decrease in T_1 to an increase in the slow motional components of water molecules, possibly secondary to enhancement of protein-water molecule interactions. Brooks et al[22] suggest that protein "water of hydration" effects are observable only at low-field strength (<0.1T). Field inhomogeneity caused by clustering of paramagnetic methemoglobin within the cells does not affect longitudinal relaxation.[23] Consequently, the mechanism responsible for the T_1 shortening noted by Gomori et al remains unclear. Additionally, it is unclear why the data do not show this reduction in T_1, although it may result from changes in the samples due to differences in the method of cell lysis (Gomori et al used freeze thaw cycles).

Direct paramagnetic effects contribute to the transverse relaxation rate, but these effects are smaller than for longitudinal relaxation. The direct paramagnetic effect was evidenced in lysed samples as the slight increase in transverse relaxation rate with increasing methemoglobin. The much larger increase seen in intact cells was due to diffusional mechanisms.

As hemoglobin is converted to methemoglobin in intact red blood cells, the paramagnetic methemoglobin becomes increasingly concentrated within the cell and a large difference in the magnetic susceptibility between the red cell cytoplasm and the surrounding plasma is generated. Thus, field gradients are created within and around the erythrocyte.[24] Diffusion of water molecules across these gradients causes the hydrogen nuclei to precess at variable rates causing irreversible dephasing and T_2 proton relax-

ation enhancement (PRE). This effect has been shown to vary as the square of the magnetic field.[21,22] Water exchange time between erythrocytes and plasma has been estimated at between 8 ms[25] and 19 ms;[26] use of short TEs does not allow time for full expression of this effect.[21,27]

The use of two echoes allows T_2* effects from the diffusion of water through local field gradients to contribute significantly to the calculated value of T_2. Estimation of T_2 using two echoes also can result in misestimation of the true value. With TE of 30 and 60 ms, substances with a long T_2 will be particularly underestimated.[14] However, any misestimation of true T_2 should not preclude the comparisons made within this paper because all samples are affected in the same manner.

Differences in the magnetic field strength used could alter the results of studies such as the current study. It is well established that T_1 increases with increasing field strength, whereas T_2 is unaffected. Paramagnetic effects, as shown with higher methemoglobin levels in lysed samples, would not be expected to change significantly with the operating field, but the observed effect might differ based on longer T_1 of the unperturbed samples. As reported previously, the diffusional effects would be expected to increase exponentially with field strength, resulting in greater disparity of T_2 between intact and lysed blood at higher methemoglobin levels.

The authors' studies of intact erythrocytes indicate a linear increase in the T_2 relaxation rate as the methemoglobin concentration rises within the cell, as has been reported previously.[4] Disruption of the cell membrane allows homogenous distribution of the paramagnetic methemoglobin throughout the solution, no field gradients are created within the solution, and the T_2 PRE should be abolished. This is consistent with the current authors' results and published reports.[21,22]

Lysis of erythrocytes alters the transverse relaxation effect of water of hydration, which has been shown to be strongly dependent on hemoglobin concentration.[28] The high concentration of hemoglobin within erythrocytes (~5 mM) impedes Brownian motion, reducing the cutoff frequency of the Brownian motion energy spectrum to about 33% of what it would be if hemoglobin were distributed uniformly throughout the blood.[24] Hemolysis reduces the "water of hydration" contribution to the transverse relaxation rate ($1/T_2$) by a factor of three. An increase in the T_2 of oxygenated blood was noted on hemolysis in the current study and in published reports.[22]

Recently it has been reported[7] that the clotting system is responsible for shortening of relaxation times. The clotting mechanism in the current study was nonphysiologic (adding thrombin to heparinized blood), but clotting did occur and thrombin was activated. The results indicated that this mechanism of clotting produced a decrease in transverse relaxation times in the 0% methemoglobin samples (normal oxygenated blood), but no statistically significant decrease was found at >94% methemoglobin. No retraction of the clots was apparent grossly at the time of imaging; presumably the bulk of the T_2 reduction was due to an intrinsic component of the clotting process rather than an increase in hematocrit. It is possible, however, that small amounts of retraction had taken place but were not yet grossly visible. No difference in T_1 was found between clotted and unclotted samples.

The authors of the current study wanted to detect the effect of clot retraction by imaging these clots 24 hours after initial imaging. However, blood gas analysis showed changes in methemoglobin content and state of oxygenation within the unclotted samples that precluded any useful comparison. The significant changes that occurred in the samples during 24 hours were complex (Table 2). Methemoglobin content decreased over time and oxygen saturation of the hemoglobin decreased. Decreased oxygen saturation implies either oxygen consumption or the production of deoxyhemoglobin. The authors speculate that the decrease in methemoglobin content may have resulted from the action of methemoglobin reductase (which would also produce deoxyhemoglobin), and metabolic activity that would result in some oxygen consumption. Regardless of the mechanism, there was significant change in the paramagnetic constituents within the sample. The degree of change suggests that it is highly important to monitor such parameters in any experiment involving aging of blood samples. Without such monitoring, precise serial interpretation of imaging characteristics would be extremely difficult.

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Announcements

4th Annual "Imaging the Head, Spine and Musculoskeletal System: A Discussion of Protocols and Applications." Presented by the Medical College of Wisconsin, February 17–22, 1991 at The Westin Kauai Resort in Kauai, Hawaii. Category 1 credit, 25 hours. Fees: $445.00 before December 31, 1990; $495.00 after December 31, 1990. Contact: Marti Carter, CME, Inc., 11011 W. North Ave, Milwaukee, WI 53226; (414) 771-9520.

Musculoskeletal Imaging for Orthopedic Surgeons and General Radiologists, March 4–8, 1991. Heywoods Resort, Barbados. Fee: $435 MDs, $300 Residents. Credits: 21. Contact: Boston University School of Medicine, Continuing Medical Education, 80 E. Concord St., Boston, MA 02118; (617) 638-4605.

Fifth Annual Magnetic Resonance Imaging Conference. Barrow Neurological Institute of St. Joseph's Hospital and Medical Center. Marriott's Camelback Inn, Scottsdale, Arizona, March 16–20, 1991. Category I CME credit. Contact: Kevin King, R.T., Education Coordinator, Radiologic Education Center, St. Joseph's Hospital and Medical Center, 350 W. Thomas Road, Phoenix, AZ 85013; (602) 285-3956.

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