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USE OF NITROXIDES AS NMR CONTRAST ENHANCING AGENTS FOR JOINTS

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NMR imaging is a well-established technology for obtaining cross-sectional anatomic pictures of organs and tissues. In addition, NMR can provide valuable information about the physiologic state of organs and tissues, especially, as a consequence of cellular injury. With this in mind, NMR in combination with gadolinium-based contrast enhancing agents has been used to assist in the detection of abnormalities to joints as well as to evaluate the status of damage resulting from an injury to this site. We describe the synthesis of a new nitroxide, which is bioresistant to the one-electron reduction mediated by superoxide in the presence of cysteine. This model mimics the reduction of nitroxides by extracellular secretion of superoxide by PMA-stimulated neutrophils. With this nitroxide, we found, in the range from 15 to 17.5 μmoles, enhancement of an NMR image in the knee joint of rabbits. Of interest is the finding that the contrast image remained for at least 90 minutes. These results demonstrate the utility of nitroxides as contrast enhancing agents for NMR imaging of joints.

KEY WORDS: Nitroxides, NMR, NMR Contrast Enhancing Agents, Spin Labels

INTRODUCTION

NMR imaging is a very important technique for obtaining cross-sectional anatomical pictures through the body of either an animal or human[1]. In addition, it can provide valuable information about the physiologic state of tissues, especially as a consequence of either pathologic conditions[2] or traumatic injuries[3]. For example, NMR imaging is used extensively to evaluate the morphology of joints, including the knee and shoulder[4]. It routinely is employed to explore for abnormalities in these joints. Most of these examinations are done without contrast enhancing agents. However, gadolium chelate-based contrast enhancing agents have found two potential applications with respect to the joints. First, when injected directly into cadaver knee joints, finer irregularities of the articular cartilage can be identified[5]. Second, when given intravenously, soft tissues around the joint such as the pannus of rheumatoid arthritis can be enhanced considerably[6].

This paper will demonstrate a third potential application of NMR contrast enhancing agents, involving the joint, namely the potential for defining differences in the rate

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of absorption of the contrast agent by the joint tissue. In so doing, inflammation or vascularity of the joint tissue should have an effect on the absorption rate and allow for the evaluation of drug therapy in degenerative joint diseases.

Of the several classes of paramagnetic compounds, inorganic ions and nitroxides have received the most attention[7,8]. Nitroxides have emerged as an exciting class of potential NMR contrast enhancing agents. Despite the obvious advantages of prolonged stability, chemical flexibility and long spin relaxation times, nitroxides are, unfortunately, rapidly bioreduced to the corresponding hydroxylamines[9,10]. Because of this limitation, we undertook an *in vivo* pharmacokinetic investigation to determine the potential use of nitroxides as NMR contrast enhancing agents[11]. We prepared over twenty new nitroxides and determined the kinetics of bioreduction in several different *in vitro* models[12]. Based on these findings, we report herein on the synthesis of a nitroxide, which appears to be resistant towards bioreduction. Furthermore, when this nitroxide is administered into the knee joint of a rabbit, a clear contrast enhancement is observed even at the low dose of 15 μmoles. Finally, we determined the distribution and the pharmacokinetic properties of this nitroxide in different regions of the knee joint.

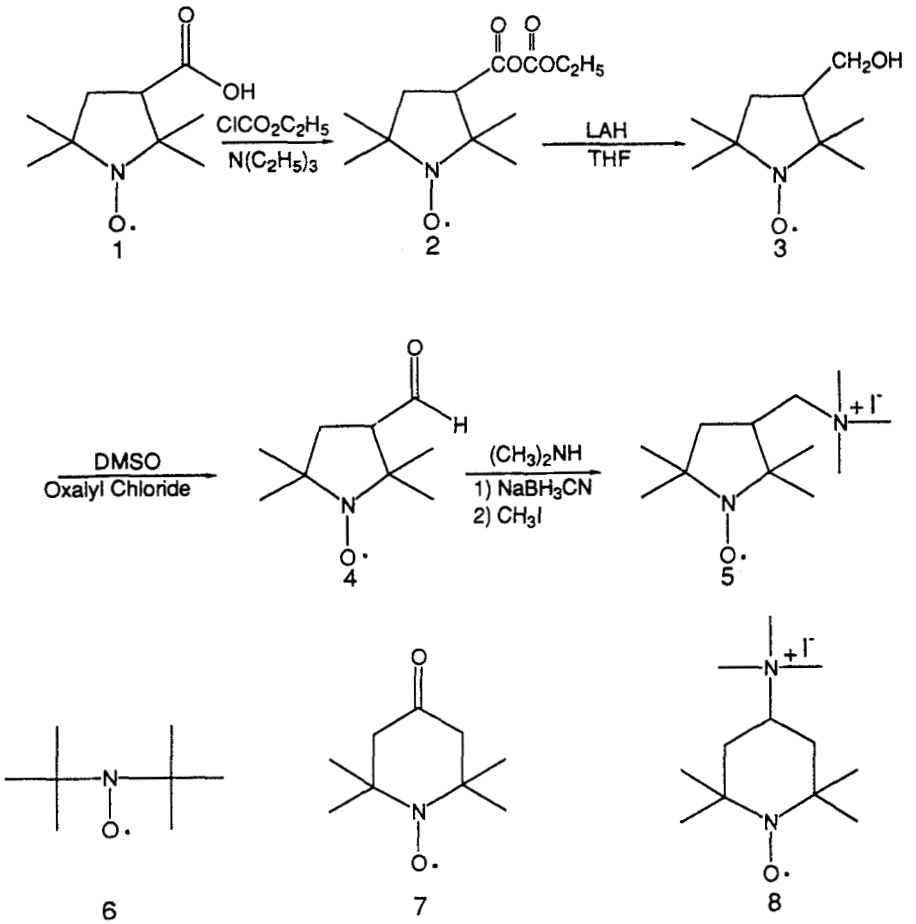
MATERIALS AND METHODS

General Comments

Diethylenetriaminepentaacetic acid (DTPA), cysteine, ferricytochrome c (type VI), phorbol 12-myristate 13-acetate (PMA) and xanthine were obtained from Sigma Chemical Company (St. Louis, MO). Xanthine oxidase and superoxide dismutase (SOD) were purchased from Boehringer Mannheim (Indianapolis, IN). Chelex-100 ion exchange resin was obtained from Bio-Rad (Richmond, CA). All buffers for the kinetic studies were passed through the resin to remove trace metal ion impurities. Di-*tert*-butylnitroxide was obtained from Polysciences (Warrington, PA). 4-Oxo-2,2,6,6-tetramethyl-1-piperidinyloxyl and 3-carboxyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl were synthesized according to methods described by Rozantsev[13], while 4-(N,N,N-trimethylamino)-2,2,6,6-tetramethyl-1-piperidinyloxyl was prepared as described in Rosen and Abou-Donia[14]. For nitroxide structures see Scheme 1.

3-Hydroxymethyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl[15,16] 3

Ethyl chloroformate (6.3 gms, 5.5 mL, 58.1 mmoles, Aldrich Chemical Company) in THF (50 mL, freshly distilled over LAH) was added slowly to a solution of 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (21) (1,10 gms, 54 mmoles) in THF (100 mL) and triethylamine (5.85 gms, 8.1 mL, 58.1 mmoles) at room temperature. The rate of the ethyl chloroformate solution addition was such that the temperature of the reaction would not exceed 20°C. Once completed, the reaction was stirred an additional hour at this temperature. At this point the reaction is filtered, the filtrate washed with additional THF and the combined THF solutions were dried over anhydrous Na_2SO_4. This solution containing the mixed anhydride was added dropwise to a suspension of LAH (3.8 gms, 100.5 mmoles) in THF (50 mL) at 0°C under argon. Once completed, the reaction was stirred an additional hour at this temperature. At this point, the reaction was quenched with water (5 mL) and was mixed overnight at room temperature in the presence of air. Upon filtering the mixture and washing extensively with



THF and ether, the combined solutions were dried over anhydrous Na_2SO_4 and evaporated to dryness, giving a yellow solid (3, 6.5 gms, 70%). A portion of which was recrystallized from ether/hexane, mp 110–112°C[16].

3-Carboxyaldehyde-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl[15] 4

The oxidation of alcohol 3 was undertaken using the method of Mancuso, *et al.*[17]. First, the DMSO/oxalyl chloride complex was formed by the dropwise addition of DMSO (5 gms, 63.8 mmoles) in CH_2Cl_2 (10 mL) to a solution of freshly distilled oxalyl chloride (4.05 gms, 2.8 mL, 31.9 mmoles, Aldrich Chemical Company) in CH_2Cl_2 (20 mL) under argon at –60°C. It was important to note that the rate of addition of DMSO did not raise the temperature of the reaction above –50°C. After the addition of DMSO was completed, the reaction was stirred for an additional 5 minutes at –60°C. Then, alcohol 3 (5 gms, 29 mmoles) in CH_2Cl_2 was added at such a rate that the temperature did not rise above –50°C. After an additional twenty minutes at –60°C, triethylamine (14.65 gms, 20 mL, 145 mmoles) in CH_2Cl_2 (20 mL) was added. During the addition of triethylamine, the temperature was not allowed to rise above –50°C. Once completed,

the reaction was stirred at this temperature for 10 minutes, then warmed to room temperature, at which point water (10 mL) was added. The CH_2Cl_2 solution was dried over anhydrous Na_2SO_4, filtered and the solution evaporated to dryness, *in vacuo*. The remaining red oil was chromatographed by flash chromatography using silica gel and hexane/ether, 7:3 ratio, which removed a small amount of orange material that was not further identified. Changing to hexane/ether, 1:2 ratio, the desired aldehyde 4 was recovered as a red oil (3.9 gms, 80%). In our hands, we found that the aldehyde is relatively unstable. Therefore, it should be stored at –20°C until needed.

3-Trimethylaminomethyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl Iodide 5

Aldehyde 4 (2 gms, 11.8 mmoles) dissolved in anhydrous methanol (10 mL) was added to a solution containing dimethyamine hydrochloride (5.76 gms, 70.6 mmoles, Aldrich Chemical Company) in methanol (50 mL) and molecular sieves (3A°). The pH of the solution was adjusted to 7–8 with triethylamine. Then, sodium cyanoborohydride (0.44 gms, 7.06 mmoles) was added to the reaction mixture, which was stirred overnight at room temperature. Upon filtration, the methanol solution was evaporated to dryness, *in vacuo*, water (100 mL) added and cooled to near 0°C. Then, dilute HCl (10%) was added until the pH was 2–3. This solution was then extracted with $CHCl_3$. The organic extract contained unreacted aldehyde 4 and alcohol 3. The remaining water solution was made basic with NaOH (10% solution) and again extracted with $CHCl_3$. The $CHCl_3$ solution was dried over anhydrous $MgSO_4$ and evaporated, *in vacuo*, to dryness, giving a yellow oil, 3-dimethylaminomethyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (1.6 gms, 70%). I.R. confirmed the absence of the aldehyde 4. Addition of methyl iodide (excess) to an ether solution of the above amine gave the methyl iodide salt 5, which was recrystallized from absolute ethanol, mp 239–240°C with decomposition.

Anal. Calc'd: C = 42.24, H = 7.68, N = 8.21, I = 37.19. Found: C = 42.20, H = 7.93, N = 8.10, I = 36.98.

Reduction of Nitroxides

The reduction rate of various nitroxides shown in Table 1 was undertaken using superoxide in the presence of a thiol, as an *in vitro* model for activated neutrophil[18], isolated as described by Borregaard, *et al.*[19]. For these experiments, the nitroxide (50 μM) was added to cysteine (200 μM) and a superoxide-generating system, consisting of xanthine (400 μM), xanthine oxidase in sodium phosphate buffer (50 mM) at pH 7.4. The rate of superoxide production was 10 μM/min as determined by following the superoxide dismutase (30 U/mL) inhibitable reduction of ferricytochrome c at 550 nm[20], using a molar absorbance of 21 mM^{-1} cm^{-1}. Nitroxide reduction rates were monitored by ESR spectroscopy at ambient temperature by observing the change of the low-field peak height of the ^{14}N-nitroxide triplet as a function of time at room temperature[12,21]. Rates of nitroxide reduction were determined according to the following equation[12,21]:

$$\text{Rate} = (1/h_o)(\Delta h/\text{min})[\text{Nitroxide}]_{\text{initial}}$$

where:

$h_o = (\Delta h/\text{min}) \times (\Delta t) + h_i$ = height at t = 0.

$\Delta h/\text{min}$ = change of height per minute.

Δt = actual time between the initiation of the reaction and the time when the scan begins as determined by a stopwatch.

h_i = height at the time when the scan is initiated.

TABLE 1
Rate of Nitroxide Reduction (μM/min)[1]

NITROXIDE	RATE[2]
1	0.52 ± 0.10
5	0.01 ± 0.01
6	4.20 ± 0.13
7	3.63 ± 0.47
8	0.17 ± 0.02

[1] Experimental details are presented in the Materials and Methods section.
[2] Each rate is the average of two independent experiments.

Partition Coefficients

Partition coefficients are defined as the ratio of the concentration of the nitroxide in 1-octanol to its concentration in HBSS, pH 7.4 buffer. Concentration in the aqueous phases were measured spectrophotometrically against standard curves before and after one hour of mixing with an equal volume of 1-octanol. The wavelength of absorption used for these determinations was 242 nm for di-*tert*-butylnitroxide 6 and 4-oxo-2,2,6,6-tetramethyl-1-piperidinyloxyl 7.

Magnetic Resonance Imaging

Male New Zealand white rabbits, weighing between 2.85 kg and 3.5 kg, were used for the NMR imaging studies. The rabbits were initially sedated with intramuscular Ketamine and Xylazine. Anesthesia was maintained with i.v. sodium pentobarbital as needed. Under fluoroscopic guidance, a needle was placed into the knee joint and 1.5 to 1.75 mL of a stock solution (10 mM) of nitroxide 5 was injected. This gave a final dose of 15 to 17.5 μmoles. The rabbit was then positioned in the MR imager. Either one or two plastic tubes containing the nitroxide at the same dose were attached to the side of the knee. These were used to act as standards and controls to demonstrate that changes in observed NMR intensity were not the result of instrumentation fluctuations, but arose due to pharmacokinetic changes in the diffusion of the nitroxide from the site of imaging.

Magnetic resonance imaging was performed with a General Electric Signa whole body MR imager operated at 1.5 Tesla. The whole body radio frequency coil was used for excitation. For reception, a 7.5 cm surface coil was placed on top of the knee. For one rabbit, this same type coil was placed above and beneath the knee and connected by a T connector to the receiving antenna port. With either experimental design, pharmacokinetic data were similar.

The pulse sequence used was a single spin echo with a TE of 20 msec and a TR of 500 msec. This is referred to as a T_1 weighed sequence. Images for quantification were obtained in the axial plane. The field of view was 8 cm with a matrix of 256 × 256. Slice thickness was 3 mm with a gap of 0.5 mm. Images were obtained at 15–20 minute intervals up to 130 minutes post-injection of the nitroxide. Images were displayed on the console and region of interest measurements of the image intensity was obtained of the fluid collections: posterior to the medial femoral condyle, posterior to the lateral condyle and lateral to the joint. Additional measurements were made with solution controls and skeletal muscles.

RESULTS AND DISCUSSION

Chemical Synthesis

The preparation of the bioreductive resistant nitroxide, 3-trimethylaminomethyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl iodide 5 is described as follows. The initial step required the synthesis of 3-carboxyaldehyde-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl 4 from 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy 1, as described in the literature[15,16]. In this two-step reaction, the ethyl carbonate 2 was prepared by slowly adding ethyl chloroformate to 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy 1 at room temperature[15]. Reduction with lithium aluminum hydride gave 3 in 70% yield. In the synthesis of 4 from 3, we found, unlike earlier studies[15], that the Swern oxidation[17] of 3 required temperatures in the range of −50°C to −60°C in order to maximize the yield of the requisite aldehyde 4. The reductive amination of 4 with sodium cyanoborohydride followed by methylation with methyl iodide gave the desired nitroxide salt 5 in reasonable yields.

Pharmacokinetic Studies

As a result of an inflammatory response, neutrophils are drawn to the site of injury. Once there, these cells become activated, at which point they undergo a rapid increase in oxygen consumption, known as the "respiratory burst"[22]. During this enhanced up-take of oxygen, superoxide and other reduction products of oxygen including, hydrogen peroxide and hydroxyl radical, in addition to hypochlorous acid, are produced to deal with the inflammatory event[23]. Not surprisingly, these reactive oxygen species can frequently mediate injury to surrounding tissue, leading in the case of joints to arthritis[24]. Although a number of antiinflammatory agents provide some relief, still our ability to be able to monitor both the effectiveness of drug therapy as well as the progression of the disease is limited. Therefore, we felt that NMR imaging in conjunction with contrast media might offer an unique opportunity to contribute to the diagnosis of inflammatory joint diseases. However, prior to initiating these studies, we must begin to determine the potential use of nitroxides as diagnostic contrast media in joints.

Evaluating the joint presents unique problems. First, in order for nitroxides to be effective NMR contrast enhancing agents for this tissue, these spin labels must be resistant to one-electron reductive processes possibly catalyzed by activated neutrophils drawn to the inflamed region. Second, diffusion of the nitroxide from this region must be sufficiently retarded to allow visualization between joint fluid and surrounding tissue. In this paper, we present our findings, documenting the ability of at least one nitroxide to meet these essential requirements, which are preliminary to the development of NMR contrast media for joints, inflammed as the result of disease or injury.

We[18,25] and others[26] have previously demonstrated that PMA (phorbol 12-myristate 13-acetate)-stimulated neutrophils rapidly reduce nitroxides to the corresponding hydroxylamines. The reaction is superoxide dependent. Therefore, our initial series of experiments were aimed at determining the rate of nitroxide reduction using an *in vitro* model to mimic activated neutrophils. For these studies, we used xanthine/xanthine oxidase, as the superoxide-generating system. Cysteine is added to the reaction mixture, since we have previously demonstrated that a thiol is required, along with superoxide, to reduce nitroxides to their corresponding hydroxylamines[25,27]. To begin, we chose di-*tert*-butylnitroxide 6 and 4-oxo-2,2,6,6-tetramethyl-1-piperidinyloxyl 7, as

representatives of lipid soluble nitroxides, having 1-octanol/water partition coefficients of 79.4 ± 10.3 and 6.9 ± 0.75, respectively. Since diffusion of the nitroxide from the joint might depend upon active transport pathways, several charged nitroxides were also included for these preliminary reduction studies. As shown in Table I, the non-charged nitroxides, 6 and 7, were rapidly reduced, while the charged nitroxides, 4-(N,N,N-trimethylamino)-2,2,6,6-tetramethyl-1-piperidinyloxyl iodide 8 and 1 were exceptionally resistant to bioreduction. In fact, nitroxide 5 was barely reduced under experimental conditions, which reduced the charged nitroxide carboxylic acid 1. These results suggest several conclusions. First, charged nitroxides, even when the charge is removed from the nitroxide, as in 5 and 8, are more resistant towards reduction than non-charged nitroxides of similar structure. Second, the rate of reduction was greater for piperidinyloxyls than pyrrolidinyloxyls. These two observations support our earlier findings using rat liver microsomes and hepatocytes[12].

Imaging Studies

From our kinetic studies, it appears that nitroxide 5 would be an ideal contrast enhancing agent for NMR imaging in the joints of rabbits. Based on a series of preliminary studies, we felt that injection of nitroxide 5 in a dose range between 15 and 17.5 μmoles into the knees of rabbits would allow us to accurately evaluate the NMR image enhancing activity of this class of contrast agents.

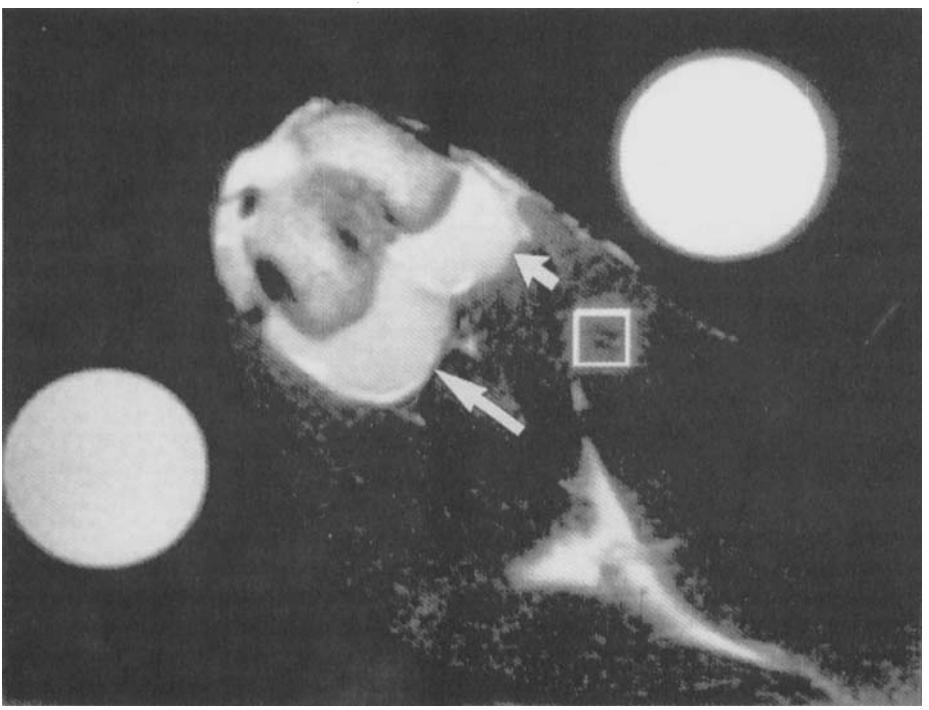


FIGURE 1A A representative axial spin echo NMR image through the knee of a rabbit is presented. The round circle on the reader's right is a standard or control tube of 10 mM stock solution of nitroxide 5. The circle on the reader's left is a standard or control tube of 1 mM stock of nitroxide 5. The arrows show intra-articular contrast fluid collections. The square region of interest is over skeletal muscle. This image was obtained two minutes after injection (15 μ moles, final dose) of the contrast enhancing agent.

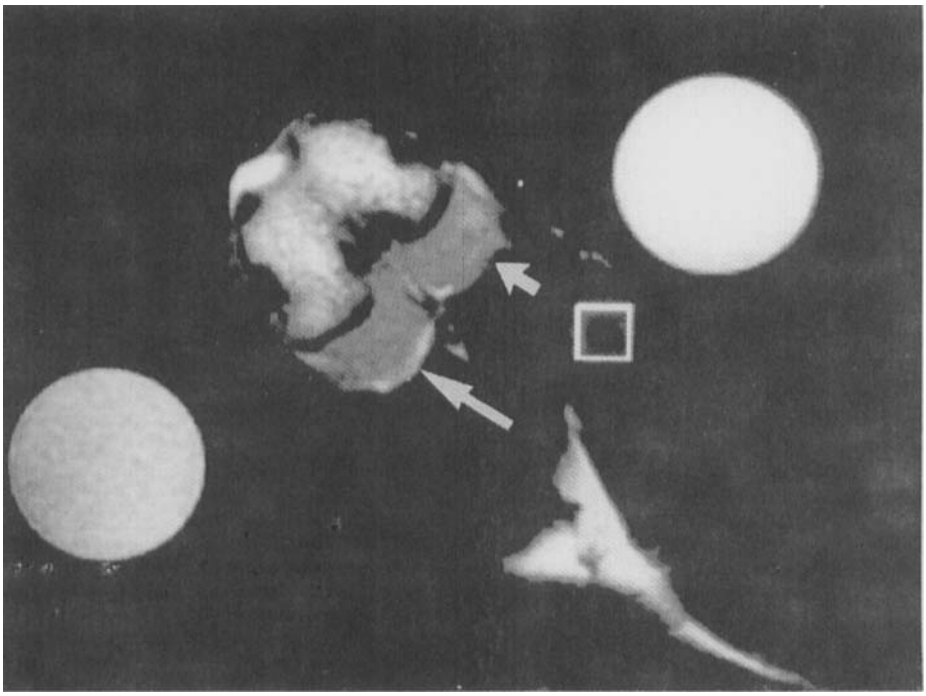


FIGURE 1B A representative axial spin echo NMR image obtained through the same rabbit knee as shown in Figure 1A at 85 minutes post-injection. The intra-articular fluid collections (note, arrows) have decreased substantially in image intensity, while the control solutions have not changed other than due to the photographic technique.

In all the experiments, the injected nitroxide distended the knee joint capsule as a homogenous fluid of increased intensity. This is demonstrated in figure 1A, which is a representative experiment of an axial NMR image of one of the rabbit knees two minutes after administration of the nitroxide. As time elapsed, the intensity of the contrast enhancement from the nitroxide decreased in intensity and volume. This is clearly demonstrated in figure 1B, which is an NMR image taken at the same location as the one in figure 1A, but obtained 85 minutes after contrast injection into the joint. The other experiments behaved similarly.

Graphs of nitroxide intensity changes, the controls and skeletal muscle as a function of time are shown in figures 2 and 3. Figure 2 demonstrates that the intensities of the nitroxide (curves 1,2, and 3) in the knee joint decreased over time. The control solutions (curves 5 and 6) change minimally as does the skeletal muscle (curve 4). In figure 3, the NMR image intensities have been divided by NMR intensities attained closest to the point of injection. This allows for relative NMR intensity changes to be measured accurately. Based on this procedure, we found that the three sampled areas of the nitroxide around the knee joint decreased similarly, suggesting a common pathway of nitroxide elimination. Alternatively, this decrease could be the result of the dilution of the nitroxide in the joint. This would seem unlikely, since the volume of the solution was also seen to decrease over time. Finally, the controls did not significantly change, demonstrating that the observed decrease in contrast enhancement was not related to fluctations in the NMR imager.

The NMR intensity change is non-linear with respect to the nitroxide concentration.

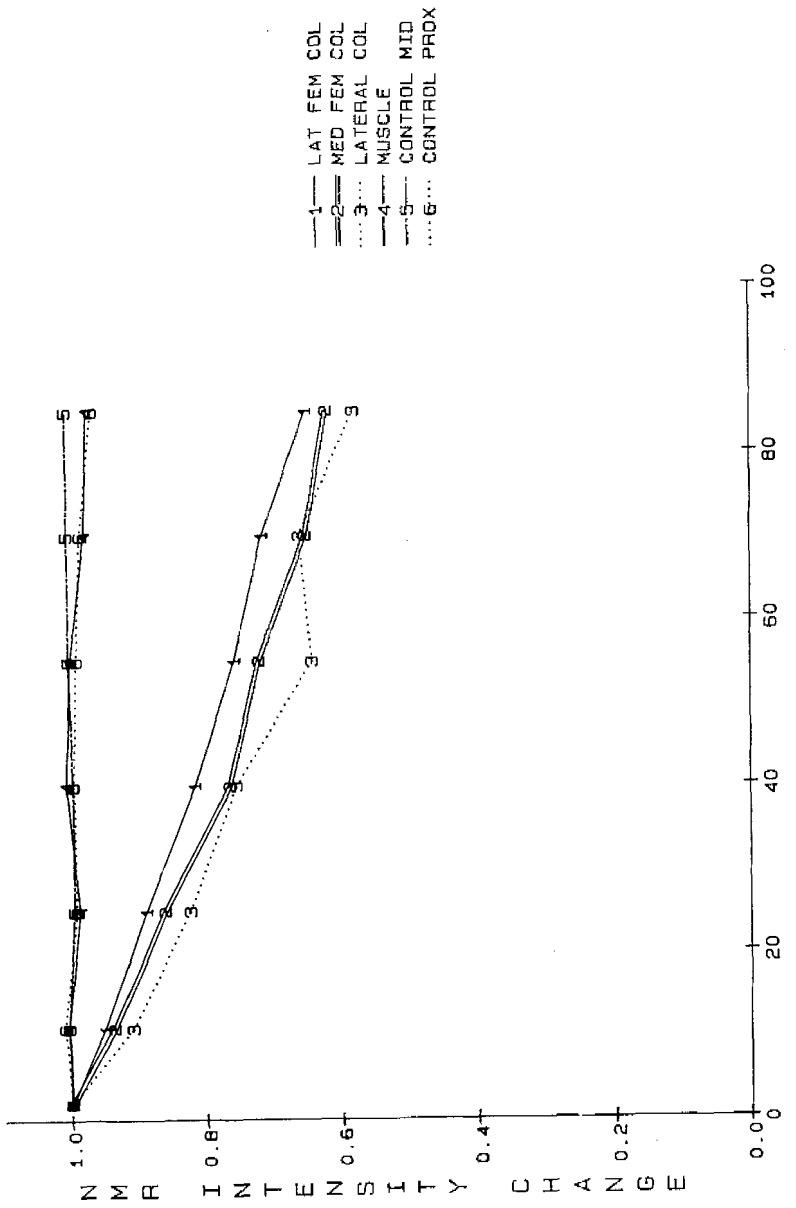


FIGURE 2 A representative graph of NMR intensity (non-standardized units) is plotted as a function of time for the fluid collections around the rabbit knee (curves 1–3), the control solutions (curves 5 and 6) and muscle (curve 4). The intensity of the contrast collections decreased with time. The control solutions and muscle do not indicate any change with time.

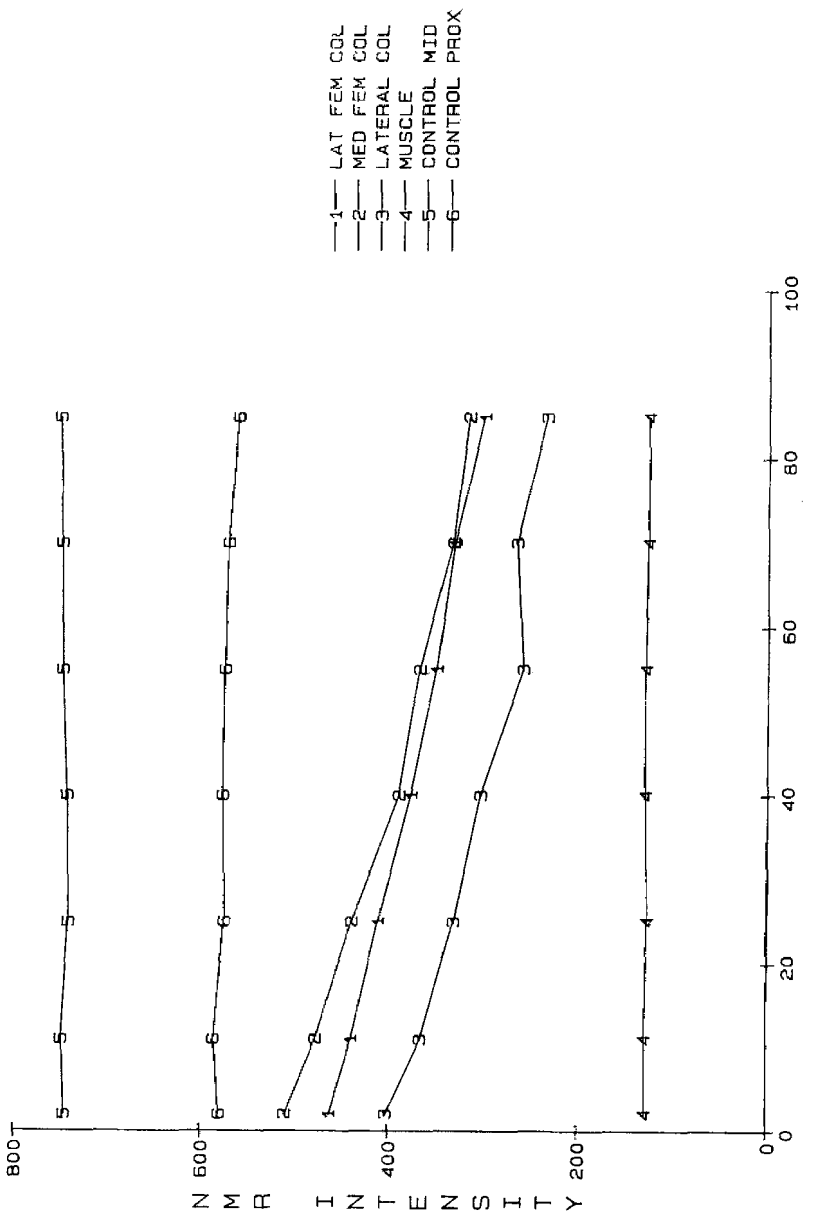


FIGURE 3 Relative NMR intensity change as a function of time, post-injection of nitroxide 2, is shown. This graph was obtained by dividing the obtained image intensity by the measurement obtained closest to the site of injection. The curves demonstrate that the contrast intensity decreased by approximately 50% over the 90 minute study as compared to controls, in which no significant change was observed.

Therefore, it is surprising that the decline in NMR intensity of the knee joint as graphed in figures 2 and 3 is nearly linear over time. This linear decrease may be due to the combined interaction of the non-linear NMR intensity change with respect to the nitroxide concentration and the presumed first order absorption of the nitroxide from the joint space. Nevertheless, rates of change in NMR intensity due to changes in NMR contrast enhancement are being used clinically in an attempt to differentiate between various pathologic conditions. To obtain reproducible numbers, which are independent of NMR equipment and pulse sequences, the NMR intensity is usually presented as a ratio against either a control solution or a control tissue, and then it is adjusted for various machine parameters[28].

Since all the sampling sites around the knee joint are part of the same fluid pool, why do they differ in NMR intensity? The non-uniformity may reflect the non-uniformity of the RF field from the application of a surface coil. This would not be a problem in human studies, since the volume RF coil is more frequently used, which results in a more uniform RF field.

The purpose of this study is to determine the feasibility of using nitroxides as potential NMR contrast enhancing agents in joints. With the development of a nitroxide with remarkable resistance towards bioreduction, we have been able to enhance the image in the knee joint of rabbits at moderate nitroxide doses. If we assume that the rate of reabsorption of the nitroxide will vary with the inflammatory status of the joint capsule, then these contrast enhancing agents may play an important role in determining degeneration of the joint and may assist in evaluting the effectiveness of specific drug therapies in combating arthritis and other inflammatory diseases.

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