Free Rad. Res. Comms., Vol. 9, No. 3-6, pp. 391-397
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INVESTIGATIONS OF THE HORSE CONCEPTUS VIA MAGNETIC RESONANCE IMAGING (MRI) AND NITROXIDE SPIN LABELS AS CONTRAST AGENTS

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Results are presented which illustrate the usefulness of Magnetic Resonance Imaging as applied to the study of living embryos. Nitroxide spin labels were employed as contrast agents to study the structure and properties of the embryos. These spin labels offer the additional advantage that they may potentially be bound to biologically important molecules thereby imparting the ability to produce contrast in the MR images to these new molecules.

The horse conceptus was chosen over other embryos due to its large size. Whereas the embryos of cattle and swine are sub-millimetre in size, the horse conceptus is on the order of 10 millimetres in diameter. The availability of microscopic imaging gradient coils will allow the techniques developed in this study to be applied to the smaller embryos of other species.

KEY WORDS: MRI, imaging, nitroxide, contrast agent, diffusion, horse, conceptus, embryo.

INTRODUCTION

Currently, projects investigating the maintenance of early pregnancy and the high incidence of early embryonic loss in the horse are being conducted by Drs. Betteridge, Savage and Burton at the University of Guelph. It was felt that Magnetic Resonance Imaging (MRI) could offer assistance in studying the transport of fluids, ions, proteins and cryoprotectants across the embryo wall. The horse conceptus possesses a double wall construction; an outer acellular capsular barrier and an inner cellular trophoblastic barrier.[1]

MRI has proven to be a useful tool for investigating the soft tissues of biological systems. The proton MR image intensities reflect proton concentrations as well as the magnetic relaxation behaviour of the protons as measured by T_1 and T_2. By appropriate weighting of these three imageable parameters, tissues can usually be distinguished by their differing image intensities.[2] These differences in intensity are referred to as contrast. Without contrast, MR imaging can yield no data.

In some situations, the tissues to be imaged cannot be distinguished because their proton concentrations as well as their T_1 and T_2 relaxation times are too similar.

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Contrast between the tissues must be generated by external means. This is usually accomplished by localizing strongly paramagnetic materials in one of the tissues. The paramagnetism of these agents alters the T_1 and T_2 values of the protons in their immediate vicinity allowing contrast to be generated. These compounds are referred to as contrast agents.

Initial MR images of the horse conceptus showed, by their low contrast, that the imageable parameters of the embryo differed only slightly from those of the surrounding medium. This investigation sought to discover whether contrast agents could yield useful information about the structure and properties of the horse conceptus. Nitroxide spin labels[3,4] were chosen as the contrast agents for this study because they can be bound easily to other molecules extending their contrast-producing properties to these new compounds. Although initial studies have used the free spin labels, future studies will focus on the behaviour of biological molecules of more immediate interest to the horse breeding industry.

MATERIALS AND METHODS

The horse conceptuses were collected by Drs. Savage and Betteridge from resident Saddlebred mares at OMAF's* Arkell Research Station. The conceptuses were presented for imaging within several hours of collection. The conceptuses were supported in either a phosphate buffered saline solution (pH 7.4) or a fetal calf serum contained within an approximately 2 1/2 inch long, 1 inch diameter pyrex tube. The tube contained a sterilized sponge support against the upper sealed end, was equipped with two side arms capped with rubber septa and a rubber stopper on the large open end. The sponge was included to support the floating conceptus away from the fluid/glass/air boundaries at the top of the cell where sharp dielectric changes produce distortions in the MR images.

The majority of MR images were recorded on an SIS 85MHz, 310 mm bore MRI spectrometer located at the University of Guelph, MRI Facility. Images were recorded as 256 by 256 point grids using a multi-slice, spin-echo imaging sequence. The repetition time was 1 second and the echo time was 35 milliseconds. For each scan, 17 parallel slices were recorded simultaneously. The high resolution images were recorded on a Bruker MSL 400 NMR spectrometer with a microscopic imaging accessory at the University of British Columbia, NMR Facility. Images were recorded as 256 by 256 point grids also using a spin-echo imaging sequence.

TEMPO and 3-carboxy-PROXYL spin labels were purchased from Aldrich Chemical Company Inc. and were used as supplied with no further purification. The phosphate buffered solution was Dulbecco's PBS, product 450-1300EH from Gibco Laboratories. The fetal calf serum was also obtained from Gibco Laboratories.

DISCUSSION

Initial spin-echo images recorded after the collection of the conceptuses show a small amount of contrast between the exterior and interior of the conceptus. No details can be seen inside the conceptus. The conceptus appears slightly darker than the sur-

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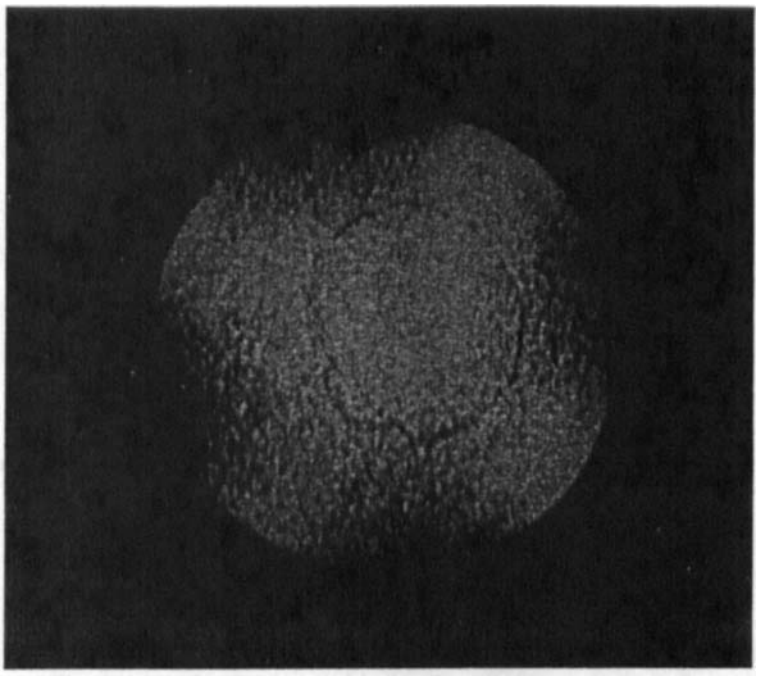

FIGURE 1 A MR image of a horse conceptus supported in fetal calf serum in the presence of Cu^{+2} ions. Image was recorded as a 256 × 256 grid using a spin-echo pulse sequence with TR ~ 4s and TE = 9 ms in a Bruker 400 MHz NMR spectrometer.

rounding aqueous medium indicating either a longer T_1 or a shorter T_2 for the interior of the embryo.[5] This distinction disappears after about one-half to one hour to yield an image with no contrast. In the absence of contrast agents, no details of the outer capsular or inner trophoblastic barriers could be distinguished by either the SIS or Bruker spectrometers.

Paramagnetic Cu^{+2} ions were added to a collapsed conceptus (one in which the acellular capsular barrier and cellular trophoblastic barrier had separated) supported in fetal calf serum and allowed to equilibrate. The Cu^{+2} ions cause an intensification of both the medium and the conceptus interior in images obtained using the Bruker microscopic imaging accessory. This indicates that the Cu^{+2} ions diffuse into the embryo's interior. A narrow dark band defining the boundary of the conceptus is seen in some of the images (Figure 1). The resolution of these images was estimated to be ~ 80 microns based on the strength of the magnetic gradients and the field-of-view used. It is felt that this dark band represents the acellular capsular barrier and that the collapsed trophoblastic barrier did not extend into the imaging plane. The SIS spectrometer, with its larger pixel size and lower gradients cannot resolve such fine detail.

Following the initial attempts to determine structural features of the conceptus, diffusion studies using nitroxide spin labels were conducted. These tests involved the TEMPO and 3-carboxy-PROXYL spin labels. TEMPO is a neutral species while 3-carboxy-PROXYL, in a pH 7.4 buffer, is approximately 99% disassociated, yielding predominately the negatively charged carboxylate ion.

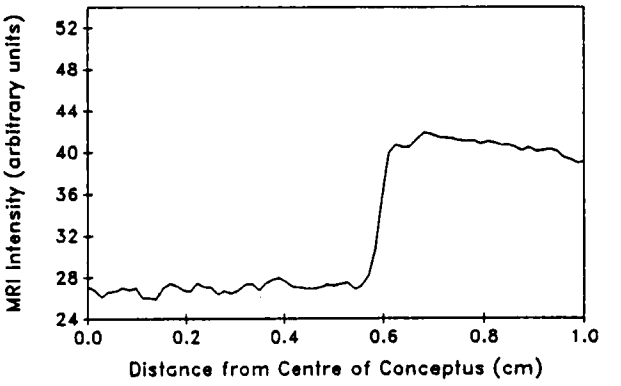

FIGURE 2 The average MR intensity profile as measured along a ray passing from the centre of the conceptus out to the surrounding medium which contained 0.002 mol/L 3-carboxy-PROXYL spin label.

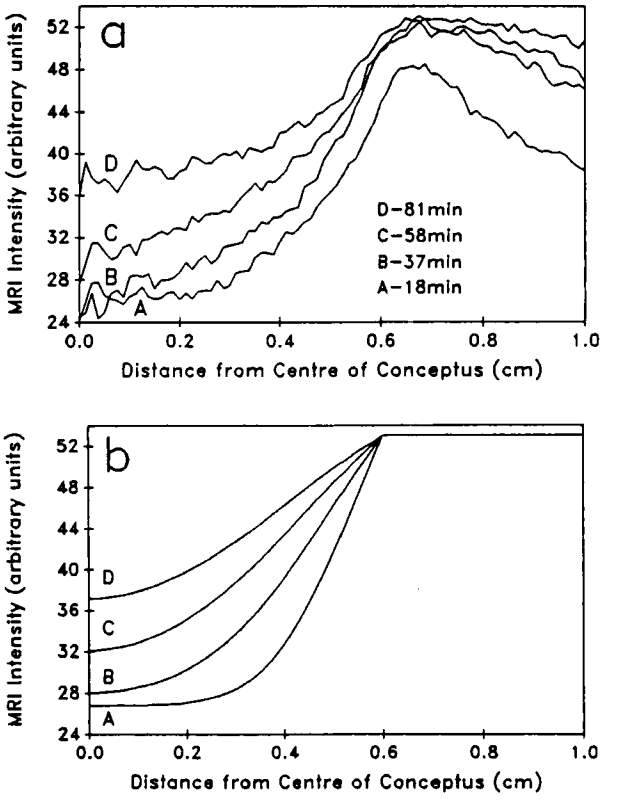

FIGURE 3 The average MR intensity profiles as measured along a ray passing from the centre of the conceptus out to the surrounding medium at various times after the introduction of a 0.004 mol/L TEMPO solution. a) Experimental curves b) Computed intensity profiles corresponding to a TEMPO diffusion coefficient of $9.0 \times 10^{-10} m^2 s^{-1}$.

When the conceptus, in phosphate buffered solution, was exposed to a 0.002 mol/L 3-carboxy-PROXYL solution for a period of 2 hours, no appreciable diffusion of the spin label into the conceptus was observed. The MR image intensity across the conceptus membrane is shown in Figure 2. It was determined by measuring the MR image intensity along 360 vectors radiating, in 1° increments, from the centre of the conceptus into the surrounding medium. The relative positions of these intensity profiles were adjusted so that the membrane crossing occurred in a constant position and the 360 profiles were then averaged.

The 3-carboxy-PROXYL solution was flushed from the imaging container using 70 ml of phosphate buffer until no contrast remained in a test image. The conceptus was then exposed to a 0.004 mol/L TEMPO solution. Intensity profiles were measured as before from the collected images and were plotted as a function of time (Figure 3a). Since the bulk of the low frequency image data is collected at the exact centre of the imaging sequence, the mid-way time is used to calculate the time-after-exposure value recorded by each profile.

In contrast to the behaviour of the negatively charged 3-carboxy-PROXYL spin label, the neutral TEMPO spin label readily diffuses into the conceptus and after 2 hours has almost completely equilibrated. To determine the diffusion coefficient of the TEMPO molecule inside the conceptus, a computer model was developed to compute the expected MRI intensity profiles. One possible complication exists since nitroxides are prone to reduction in some biological systems. This reduction converts the paramagnetic radicals to diamagnetic hydroxylamines which have no contrast producing ability. Although TEMPO is reported to reduce more easily than five-membered ring spin labels[6] there is no evidence that reduction is occurring in this system. After two hours exposure to the TEMPO solution, the MR image shows virtually no contrast indicating a near-uniform TEMPO concentration throughout the conceptus and the surrounding solution. Because the TEMPO concentration inside the conceptus is not significantly lowered, any reduction which is occurring must be slow in comparison to the rate of diffusion or may have been exhausted early in the experiment.

For the computer simulation, the conceptus was modelled as a number of concentric spherical shells. Using the expression;

$J = -D(\delta c/\delta x)$ where J is the flux of material in mol/(s·m^2), D is the diffusion coefficient in m^2/s and $\delta c/\delta x$ is the concentration gradient in mol/m^4.

the amount of material crossing each boundary between two shells per time increment was calculated as;

$\Delta mol = J \cdot A \cdot \Delta t$ where Δmol is the number of moles crossing the boundary, A is the area of the boundary and Δt is the time increment of the simulation.

Finally, the concentrations of the contrast agent were calculated and converted to MRI intensity enhancements using the experimentally determined plot of MRI intensity enhancement ($I_{\text{with contrast agent}}/I_{\text{without contrast agent}}$) versus TEMPO concentration (Figure 4). This conversion accounts for the non-linear relationship between TEMPO concentration and MRI intensity. At 0.004 mol/L, the MRI intensity approximately doubles.

The shape of the curves calculated by the simulation (Figure 3b) deviate somewhat

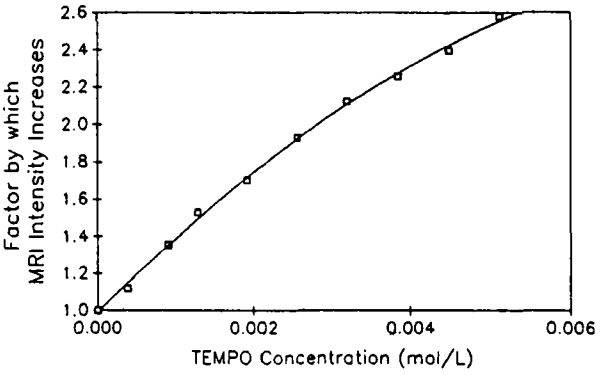

FIGURE 4 Plot of MRI intensity enhancement versus concentration of TEMPO in PBS buffer solution using a spin-echo pulse sequence, TR = 1 s and TE = 35 ms.

from the experimental results because the conceptus is not a true sphere as assumed, and in fact is somewhat flattened.

Comparison of calculated and experimental MRI intensity profiles across the conceptus barriers suggest that the diffusion constant for TEMPO inside the conceptus is $9.0 \pm 0.5 \times 10^{-10} m^2/s$. Diffusion constants for some spin labels and spin labeled compounds have been reported.[7,8] Of particular interest for comparison are the diffusion constants of 7.6×10^{-10} and $0.64 \times 10^{-10} m^2/s$ reported for 4-hydroxy-TEMPO[9] in decalin and squalane respectively.

CONCLUSIONS

The results presented here suggest that it is indeed possible to image structural details as fine as the conceptus barrier. Optimal conditions for doing this have not yet been found. Improved methods for localizing the conceptus should yield sharper images of the conceptus wall. Attempts to distinguish the capsular and trophoblastic barriers in collapsed conceptuses by MRI appear feasible.

Of more immediate usefulness, is the ability of MRI to detect the presence of nitroxide spin labels through their contrast enhancing properties. MRI can detect not only the absence or presence of these spin labels inside the conceptus but can also yield concentration profiles with distance from the conceptus wall. Our preliminary results show that appropriate analysis of the image data can yield rates of diffusion of molecules within the conceptus. Using covalently bonded spin labels, molecules of biological interest can be studied by the same technique.

Also of interest is the fact that the neutral TEMPO molecule and the positively charged Cu^{+2} ions are observed to penetrate both conceptus barriers. The negatively charged 3-carboxy-PROXYL spin label, on the other hand, is excluded from the conceptus. This suggests that one or more of the conceptus barriers may possess a repulsive negative charge. Studies of additional charged nitroxides will show whether the exclusion is truely charge related and which of the two barriers are involved.

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Accepted by Prof. B. Halliwell