Successful and safe perfusion of the primate brainstem: in vivo magnetic resonance imaging of macromolecular distribution during infusion

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Object. Intrinsic disease processes of the brainstem (gliomas, neurodegenerative disease, and others) have remained difficult or impossible to treat effectively because of limited drug penetration across the blood–brainstem barrier with conventional delivery methods. The authors used convection-enhanced delivery (CED) of a macromolecular tracer visible on magnetic resonance (MR) imaging to examine the utility of CED for safe perfusion of the brainstem.

Methods. Three primates (Macaca mulatta) underwent CED of various volumes of infusion ([Vis]; 85, 110, and 120 μL) of Gd-bound albumin (72 kD) in the pontine region of the brainstem during serial MR imaging. Infusate volume of distribution (Vd), homogeneity, and anatomical distribution were visualized and quantified using MR imaging. Neurological function was observed and recorded up to 35 days postinfusion. Histological analysis was performed in all animals. Large regions of the pons and midbrain were successfully and safely perfused with the macromolecular protein. The Vd was linearly proportional to the Vi (R² = 0.94), with a Vd/Vi ratio of 8.7 ± 1.2 (mean ± standard deviation). Furthermore, the concentration across the perfused region was homogeneous. The Vd increased slightly at 24 hours after completion of the infusion, and remained larger until the intensity of infusion faded (by Day 7). No animal exhibited a neurological deficit after infusion. Histological analysis revealed normal tissue architecture and minimal gliosis that was limited to the region immediately surrounding the cannula track.

Conclusions. First, CED can be used to perfuse the brainstem safely and effectively with macromolecules. Second, a large-molecular-weight imaging tracer can be used successfully to deliver, monitor in vivo, and control the distribution of small- and large-molecular-weight putative therapeutic agents for treatment of intrinsic brainstem processes.

KEY WORDS • brainstem • convection-enhanced delivery • magnetic resonance imaging • Macaca mulatta • rat

Intrinsic diseases of the brainstem often result in serious morbidity or death because there is no effective surgical or medical therapy. Although a growing number of potentially therapeutic compounds exist for treating these disorders, inadequate delivery of these agents to this region limits their effective use. Currently available brainstem delivery methods rely on systemic or intrathecal drug administration, both of which have a number of inherent limitations. Systemic delivery is frequently restricted by systemic toxicity and the inability of many compounds to cross the blood–brainstem barrier. Also, even if systemically delivered agents cross the blood–brainstem barrier, their distribution is either heterogeneous or non-targeted. Penetration into the brainstem after intrathecal delivery relies on diffusion, which severely constrains tissue distribution and which also produces nontargeted, heterogeneous dispersion throughout the CNS. Because of the limitations of these delivery methods, potentially therapeutic substances have yet to be effective in the treatment of intrinsic diseases of the brainstem.

To overcome the restrictions associated with currently available delivery methods, we investigated the use of CED to distribute a macromolecular MR-visible tracer (Gd-bound albumin; 72 kD) within the brainstem. Because many of the most promising putative therapeutic agents, such as antibodies, gene vectors, and immunotoxins, are macromolecules (molecular weight > 10 kD), an ideal delivery model would allow for the successful distribution of large-molecular-weight substances in a safe, reliable, and homogeneous manner over large anatomical...
Preparation of Gd-Bound Albumin

The 1B4M-DTPA was conjugated to HSA by modification of a previously described method. Briefly, 100 to 150 mg of HSA was dissolved in 20 ml of 50 mM sodium bicarbonate and 0.15 M NaCl at pH 8.5. To this solution, 45 mg of 1B4M-DTPA dissolved in 1 ml H2O (initial ratio of ligand/HSA, 30) was then added. The reaction mixture was rotated at room temperature overnight. The unreacted or free ligand was then separated from the HSA conjugate by centrifugation. The final ligand/HSA ratio was then determined spectrophotometrically. The final volume of the purified HSA–1B4M-DTPA was adjusted to deliver a concentration of approximately 10 mg/ml HSA.

To determine whether the physical properties of convective delivery permit effective drug distribution in the brainstem, to examine the potential for monitoring the distribution of infusate in the brainstem during infusion, and to examine the safety of perfusion of the brainstem with CED, we used this method to distribute Gd-bound albumin in the brainstem of nonhuman primates during in vivo, real-time MR imaging.

Materials and Methods

Preparation of Gd-Bound Albumin

The 1B4M-DTPA was conjugated to HSA by modification of a previously described method. Briefly, 100 to 150 mg of HSA was dissolved in 20 ml of 50 mM sodium bicarbonate and 0.15 M NaCl at pH 8.5. To this solution, 45 mg of 1B4M-DTPA dissolved in 1 ml H2O (initial ratio of ligand/HSA, 30) was then added. The reaction mixture was rotated at room temperature overnight. The unreacted or free ligand was then separated from the HSA conjugate by centrifugation. The final ligand/HSA ratio was then determined spectrophotometrically. The final volume of the purified HSA–1B4M-DTPA was adjusted to deliver a concentration of approximately 10 mg/ml HSA.

The Gd (III) was then reacted with the HSA–1B4M-DTPA at an initial 2:1 molar ratio (Gd (III)/1B4M-DTPA) by using a standard solution of Gd (III) (Gd(NO3)3, 6.42 × 10−3 M). The pH of the Gd (III) solution was adjusted to between 4.5 and 5 by using 5 M NH4OAc, and the solution was added to HSA–1B4M-DTPA by drops while mixing the reaction. The mixture was allowed to continue for 5 to 6 hours at room temperature with rotation. The unreacted Gd (III) was removed by adding 0.5 ml of 0.1 M ethylenediamine tetraacetic acid solution, followed by centrifugation. The final concentration of albumin was determined spectrophotometrically by measuring the absorbance at 280 nm. The percentage of Gd (III) incorporation was determined by repeating the measurement of the number of chelating agents on the protein and noting the decrease resulting from their occupation by Gd (III). Each HSA molecule was linked to five Gd molecules. A stock solution of the Gd-bound albumin (28 mg/ml) in phosphate-buffered saline was then infused into the animals.

Toxicity Trial of Gd-Bound Albumin in Rats

All animal investigations were conducted in accordance with the National Institutes of Health guidelines on the use of animals in research and were approved by the Animal Care and Use Committee of the National Institute of Neurological Disorders and Stroke. Twelve adult male Sprague–Dawley rats weighing between 300 and 400 g each were anesthetized with 2% isoflurane and placed in a stereotactic frame. A sagittal incision was made through the scalp to the level of the skull, and a burr hole was placed over the right frontal region. A 32-gauge cannula attached to a 25-μl Hamilton syringe filled with Gd-bound albumin was stereotactically placed in the right striatum. The coordinates for placement of the cannula tip in the striatum were 0.5 mm anterior to the bregma, 2.8 mm right of the midline, and 5 mm below the level of the dura matter.

To distribute the infusate by using convection, we used a noncompliant, gas-tight infusion apparatus that has been described in detail previously. It consists of a syringe pump that generates a continuous pressure gradient, which is transmitted through a hydraulic drive attached to the infusate syringe plunger. Using this system, 10 μl of Gd-bound albumin was delivered at a rate of 0.5 μl/minute into the striatum.

After completion of the infusion, the animals were allowed to awaken. They were observed daily for clinical deficits, and were killed at the end of the observation period (3–60 days). After the animals had been killed, their brains were immediately removed and frozen at −70°C. The brains were then cut coronally into 20-μm–thick serial sections on a cryostat at −18 to −20°C; tissue sections were cut through the entire brain. These sections were stained with hematoxylin and eosin, Luxol fast blue, and Nissl, and immunohistochemical investigation for GFAP was then performed.

The CED of Gd-Bound Albumin in Primates

Three adult primates (Macaca mulatta) underwent CED of Gd-bound albumin to the pontine region of the brainstem. After anesthesia had been induced in the animals, they were intubated and general endotracheal anesthesia was induced with halothane. The animals’ temperature, heart rate, oxygen saturation, electrocardiographic responses, and end-tidal PCO2, were monitored. The head of each animal was then secured in a stereotactic frame. Using sterile methods, a midline skin incision was made from the anterior to the posterior aspect of the vertex of the animal’s skull, and self-retaining retractors were placed to position and expose the underlying skull. A 1-cm burr hole was placed over the stereotactically determined entry point, and the underlying dura mater was incised. The outer guide cannula (outer diameter 0.027 in, inner diameter 0.02 in) was stereotactically placed through the dural opening along the target trajectory to a level 1.5 cm above the desired pontine target. The guide cannula was then secured in place with methylmethacrylate. The inner cannula (outer diameter 0.014 in, inner diameter 0.006 in), after being connected to the infusion apparatus, was placed through the outer guide cannula to the target (Fig. 1). The animal was then secured in the MR imager for imaging studies during infusion.

To distribute infusate into the brainstem by using convection, we used a noncompliant delivery system that is gastight with no dead volume. A Harvard syringe pump was used to generate continuous pressure throughout the infusion procedure. During infusion, the pressure was transmitted from the pump to a glass, gastight, infusate-filled Hamilton syringe (250 μl total volume) that was connected to PEEK tubing (outer diameter 0.23 in, inner diameter 0.05 in). The PEEK tubing was attached directly to the inner infusion cannula that was placed directly into the pontine region of the brainstem. Infusions were performed at rates between 0.25 and 1 μl/minute.

In Vivo MR Imaging of Gd-Bound Albumin in Primates

Real-Time Imaging. After placement of the infusion cannula, coronal T1-weighted MR images were obtained to determine the precise location of the inner cannula. Once cannula placement was confirmed, infusions were started and T1-weighted MR images were obtained in three planes (sagittal, axial, and coronal; slice thickness 1–1.5 mm, 0 mm spacing) by using a 1.5-tesla imager at approximately 20- to 40-minute intervals until the infusions were complete. The MR images were analyzed on a Sun workstation. Three-dimensional Vds were calculated using a threshold for segmentation as the signal intensity value two SDs above the mean baseline signal from the surrounding noninfused anatomical region. To determine the homogeneity of the infusion over the infused Vd, line profiles were obtained through the center of the infusion as seen on coronal images. The validity and accuracy of these methods for calculating Vd and homogeneity from MR imaging have been confirmed previously by using quantitative autoradiography (TT Nguyen, et al., unpublished data).

Postinfusion Imaging. Two primates also underwent MR imaging on Days 0, 1, 2, 4, and 7 after infusion of the Gd-bound albumin. The two animals, T1-weighted MR images were obtained in three planes by using a 1.5-tesla imager (slice thickness 1 mm, 0 mm spacing). The three-dimensional Vd was calculated and the homogeneity of the infusion was determined as described previously.
Neurological Observation of Primates

Animals were observed daily for medical or neurological difficulties during the study period (18–35 days). Postoperative videotaping was performed in each animal within 48 hours of infusion completion and within 24 hours of death.

Histological Analysis of the Brainstem

Animals were killed by overdoses of barbiturates at the completion of the clinical observation period. Immediately afterward, the animals’ brains were perfused with phosphate-buffered saline followed by 10% formalin. The brainstems were then cut coronally into 20-μm-thick serial sections. Tissue sections cut through the entire brainstem were processed for histological review, and representative sections from each tissue block were stained with hematoxylin and eosin, Luxol fast blue, and Nissl. Immunohistochemical studies for GFAP were performed.

Statistical Analysis

Statistical analysis was performed using commercially available software, and specific statistical tests were used as defined in the text.

Sources of Supplies and Equipment

The HSA was acquired from Sigma Chemical Co., St. Louis, MO. The Gd (III) was obtained from Aldrich, Milwaukee, WI. The syringe pump (model 22) was obtained from Harvard Apparatus, Inc., South Natick, MA. The Hamilton syringe and PEEK tubing were provided by Thompson Instruments, Springfield, MA. The silica cannulas and methylmethacrylate were obtained from Plastics One, Roanoke, VA. The stereotactic frame (model 1504) was obtained from Kopf Instruments, Tujunga, CA. The Sun workstation and software were purchased from Sun Microsystems, Inc., Palo Alto, CA. The Statview 5.0 software was purchased from Abacus Concepts, Inc., Berkeley, CA.

Results

Preparation of Gd-Bound Albumin

To create a large-molecular-weight marker for MR imaging, we developed a method for the stable chelation of Gd to albumin. Although Gd is an excellent marker for MR imaging, it is neurotoxic in its free state. Thus, it was critical to develop a stable construct in which the Gd would be permanently bound to albumin. After synthesis, the Gd-bound albumin remained soluble, did not aggregate, and remained completely bound.

Toxicity Trial of Gd-Bound Albumin in Rats

Before infusing the Gd-bound albumin in primates, we confirmed the safety of this compound in 12 rats. Each rat underwent unilateral CED of 10 μl of Gd-bound albumin to the striatum. None of the animals exhibited clinical deficits after extended observation (up to 60 days), and histological analysis revealed normal tissue architecture with minimal gliosis that was limited to the immediate region surrounding the infusion cannula (within a 25-μm radius of the cannula) after infusion.

Real-Time MR Imaging of Gd-Bound Albumin Delivery in Primates

To assess the feasibility of distributing to the brainstem a clinically relevant volume of a large molecule while using MR imaging to monitor its distribution in vivo, we obtained MR images in three primates (Macaca mulatta; Table 1) during convective delivery of Gd-bound albumin.
to the pontine region. Real-time imaging performed at 20- to 40-minute intervals during infusion delivery demonstrated that the anatomical region infused with Gd-bound albumin was clearly distinguishable from the surrounding noninfused tissue (Fig. 2). The pontine region surrounding the tip of the cannula steadily filled with the increasing Vi until the surrounding anatomical region was nearly filled with infusate (Figs. 3 and 4).

Volumetric analysis of the infused region at various time points during the infusion revealed that the Vd of the Gd-bound albumin increased linearly with the Vi ($R^2 = 0.94$; Fig. 5) and the Vd/Vi ratio over the volumes infused was $8.7 \pm 1.2$ (mean $\pm$ SD; Figs. 5 and 6). The concentration of the delivered infusate in tissue appeared to be homogeneous. Cross-sectional line profiles drawn through the infused region formed a square pattern that was indicative of a relatively uniform concentration over the region, with a sharp gradient drop-off at the edges (Fig. 7).

**Postinfusion MR Imaging of Gd-Bound Albumin in Primates**

To determine the characteristics of the infusion at time points after its completion, we performed MR imaging in two of the primates at Days 1, 2, 4, and 7 postinfusion. Results of MR imaging of the infused region revealed an increase in Vd (compared with the Vd observed immediately after completion of the infusion) starting at postinfusion Day 1 (Fig. 8). The postinfusion Day 1 increase in Vd

![Fig. 3. Real-time T1-weighted MR images obtained in the coronal (A) and midsagittal planes (B-H) at various time points during the infusion of Gd-bound albumin in Animal 1 (total Vi 85 μl).](image)

![Fig. 4. Real-time T1-weighted MR images obtained in the coronal plane at various time points during the infusion of Gd-bound albumin in Animal 3 (rate of infusion 0.5 μl/minute, total Vi 120 μl).](image)
was 35% for Animal 2 and 41% for Animal 3. These increases remained relatively stable until the intensity of Gd-bound albumin faded to that of the surrounding normal tissues (by postinfusion Day 7; Fig. 8). Cross-sectional line profiles drawn through the infused region on MR imaging (postinfusion Days 1 and 2) continued to have a square pattern (data not shown), which was indicative of a continued uniform concentration in the region of infusion.

Clinical and Histological Effects of the CED of Gd-Bound Albumin in Primates

To determine the safety and potential for tissue toxicity of the CED of Gd-bound albumin in the brainstem, the primates subjected to infusion were followed with serial clinical examinations (up to 35 days), and histological analysis of the infused region was performed after the animals were killed. Throughout the observation period, no animal had a detectable neurological deficit after infusion. Gross examination of the brain and brainstem revealed normal weight and no evidence of edema. Brainstem sections prepared with Luxol fast blue, Nissl, and hematoxylin and eosin stains revealed normal tissue architecture in the infused region (Fig. 9). Use of GFAP staining revealed minimal gliosis that was limited to the region immediately surrounding the cannula track (within approximately a 25- to 50-μm radius of the cannula).

Discussion

The CED Method

The CED method relies on bulk flow that is driven by a small pressure gradient to distribute substances within the interstitial spaces of the CNS. Unlike intraventricular or intrathecal delivery, which relies on diffusion, convection is not limited by the infusate’s molecular weight, concentration, or diffusivity. Moreover, because convective delivery directly distributes molecules within the parenchyma, it can be used to target selected regions of the CNS in a manner that bypasses the blood–brainstem barrier, which limits the distribution and efficacy of systemically delivered agents. In previous studies in our laboratory it has been shown that the properties of convective delivery can be used to distribute small and large molecules homogeneously over clinically relevant volumes in a safe and reproducible manner within the spinal cord and brain. In this study, we used CED of a macromolecular MR tracer for in vivo real-time monitoring of infusate distribution within the primate brainstem.

Current Study

Real-Time MR Imaging. During the infusion of Gd-bound albumin in primates, MR images of the discrete advancing edge of the wavefront of perfused tissue obtained at 20- to 40-minute intervals permitted the anatomical region infused with Gd-bound albumin to be clearly distinguished from the surrounding noninfused tissue (Figs. 2–4). The pontine region surrounding the tip of the cannula filled steadily with the increasing Vi until the entire anatomical region was nearly filled with infusate (Figs. 3 and 4). These findings not only reveal the potential of this method for perfusion of the brainstem on a clinically relevant scale, they also indicate the value of real-time, noninvasive monitoring of infusate distribution. The accuracy of determining anatomical distribution and Vd by using this surrogate MR-visible tracer has been demonstrated previously by quantitative autoradiography (TT Nguyen, et al., unpublished data).

The Vd/Vi Ratio. The volume of tissue perfused (that is, the Vd) increased linearly (R² = 0.94) with increasing Vi.
The overall Vd/Vi ratio was 8.7 ± 1.2. This ratio reflects the distribution of substances within the interstitial spaces and is higher than those in previous studies, in which the CED of similar substances within the gray matter of the brain (Vd/Vi 5 ± 0.2)² and the white matter tract of the brain² and spinal cord (Vd/Vi 4.4 ± 0.5) was examined.¹⁰ This indicates that the interstitial space of the brainstem is small compared with these other regions of the CNS, because distribution is inversely related to the size of the extracellular space of the infused region. A reduction of

![Image](image_url)

**Fig. 7.** *Upper:* Coronal T₁-weighted MR image obtained in Animal 3 with cross-sectional line profile (red line drawn through infused region). *Lower:* Graph of line profile demonstrating the square pattern indicative of relatively uniform concentration over the region of infusion, and a sharp gradient drop-off at the edges.
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interstitial space is not surprising considering the densely packed white matter tracts that exist in this region of the CNS. The tight arrangement of fibers in this region and the large Vd/Vi ratio permit rapid filling of clinically relevant portions of tissue.

It should be noted that our findings differ from the recent report of Sandberg, et al., who examined the safety of CED of fluorescein isothiocyanate–dextran (20 kD) into the rat brainstem. Although they demonstrated in that study that in a small animal a portion of brainstem can be safely perfused using CED, only a small portion of the pons was perfused (Vi 0.5–4 μl). Furthermore, they found substantially larger Vd/Vi ratios (mean 14–30.9) than those shown in previously published studies (mean 4.4–5.4)5,10 and that reported in this study (mean 8.7). The discrepancy in the Vd/Vi ratio may be explained by their use of fluorescein isothiocyanate–dextran and their inability to define the margin of the perfused region by using ultraviolet illumination of the fluorescence-labeled infusate, the accuracy of which has not been confirmed using any established method. Furthermore, the use of a fluorescence-labeled infusate prevented any qualitative or quantitative assessment of concentrations across the region perfused.

Safety and Histological Findings. The CED of Gd-bound albumin was well tolerated, with no evidence of neurological injury in the rat and primate models. In none of the animals was there evidence of neurological deficits after infusion at any time during the observation period (up to

![Fig. 8.](image_url)
60 days). Histological analysis of infused tissue was consistent with these clinical findings. Serial tissue sections cut through the region of infusion revealed only minimal gliosis in the area immediately surrounding the cannula track (within a 25- to 50-μm radius of the cannula) and the absence of tissue injury throughout the entire perfused area in both rats and primates (Fig. 9). These findings are consistent with previous ones that have shown that convective distribution of molecules over relatively large volumes in the CNS can be performed safely, and can be achieved without significant elevation of interstitial pressure in normal or tumor tissues at the delivery rates used in this study. Conceivably, additional measures that could be used to enhance the safety of brainstem perfusion in the clinical setting may include electrophysiological monitoring (for example, of somatosensory evoked potentials and/or brainstem evoked potentials) during infusion.

**Postinfusion Distribution of Infusate.** The performance of MR imaging during and after infusion permitted assessment of the Vd of Gd-bound albumin after completion of the infusion. It revealed expansion of the Vd on postinfusion Day 1, and no further expansion on subsequent imaging. This behavior is consistent with the limited diffusional spread of Gd-bound albumin after the end of infusion. Previously, it has been calculated that a macromolecule similar in size to Gd-bound albumin and cleared with a half-life of 33 hours will diffuse an additional millimeter before clearance drops its concentration below a few percentages of the initial infusate concentration. The additional volume from this small expansion of the diffusional shell to the Vd existing at Day 0 (Fig. 8) leads to a computed volume increase of approximately 40 to 55%, a figure similar to the 35 and 41% increases observed experimentally. Because of ongoing local clearance of Gd-bound albumin, maximal diffusional spread is limited and results in stabilization of the size of the infused area found in this study on more distant images (postinfusion Day 2; Fig. 8). Although it is conceivable that viscoelastic changes may also be occurring in the postinfusion period and contributing to Vd expansion, they do not appear to be quantitatively more important than the simple diffusional contributions. Although this small additional shell increased the Vd significantly in the current study at infusion levels of 120 μl, it should occur at the same absolute distance with much larger infusions (we have previously safely delivered volumes as high as 240 ml in humans with tumors), and in that setting will comprise a rapidly diminishing fraction of the total perfused volume.

**Homogeneity of Infusion.** The distribution of the infusate appeared to be homogeneous; analysis of infusate intensity on MR imaging revealed uniformity and a sharp drop-off at the edges of the infused region. This square concentration profile was maintained throughout the period of strong visible enhancement on postinfusion MR imaging (through postinfusion Day 2; Fig. 7). These findings are consistent with the bulk flow properties of CED and with a number of previous studies, in which convective delivery of macromolecules (including 14C-albumin) to the CNS was used. These results also underscore the ability of this method to distribute molecules in a highly uniform concentration over the targeted region, and to maintain this uniformity during an extended period of time.

**Potential Applications**

The CED of macromolecules to the brainstem should have widespread applications in the investigation and treatment of brainstem conditions. One example of a specific lesion that might ideally be treated with CED of therapeutic agents is that of diffuse pontine gliomas. These neoplasms primarily affect young children and are uniformly fatal because they are not surgically accessible and because chemotherapeutic agents cannot be delivered using conventional methods due to the limitations of these methods.

The potential of CED to distribute therapeutic molecules effectively to brainstem tumors and the ability to monitor the distribution of infusate noninvasively in real time (including the pattern of distribution, the Vd, and the...
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anatomical location of infusate) should prove very useful in such cases. Such monitoring may be critical because of important alterations in tissue properties among individual patients in the setting of infiltrative tumor cells and surrounding edema. Ideally, the therapeutic compound being infused would have distribution characteristics similar to the confounded surrogate tracer (Gd-bound albumin), so that distribution could be precisely determined using MR imaging. Subsequently, small (< 600-D), highly lipophilic compounds (for example, nitrosoureas), which are not well suited for CED because of rapid extravasation from the tissue into tumor and normal capillaries, may not be accurately monitored by this method unless they can be directly tagged with an imaging marker.

Conclusions

The results of this study indicate that CED can be safely used to perfuse large, clinically relevant portions of the primate brainstem. The ability to confuse surrogate imaging tracers or directly label therapeutic compounds allows real-time visualization of drug delivery into this and other regions of the CNS. Thus, regional delivery in which CED is used in this manner should prove valuable in studying and treating brainstem gliomas and a variety of other CNS disorders.

References


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