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Safety of real-time convection-enhanced delivery of liposomes to primate brain: a long-term retrospective

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Abstract

Convection-enhanced delivery (CED) is gaining popularity in direct brain infusions. Our group has pioneered the use of liposomes loaded with the MRI contrast reagent as a means to track and quantitate CED in the primate brain through real-time MRI. When co-infused with therapeutic nanoparticles, these tracking liposomes provide us with unprecedented precision in the management of infusions into discrete brain regions. In order to translate real-time CED into clinical application, several important parameters must be defined. In this study, we have analyzed all our cumulative animal data to answer a number of questions as to whether real-time CED in primates depends on concentration of infusate, is reproducible, allows prediction of distribution in a given anatomic structure, and whether it has long term pathological consequences. Our retrospective analysis indicates that real-time CED is highly predictable; repeated procedures yielded identical results, and no long-term brain pathologies were found. We conclude that introduction of our technique to clinical application would enhance accuracy and patient safety when compared to current non-monitored delivery trials.

Introduction

Convection-enhanced delivery (CED) was first described by Bobo et al. in 1994 (1), and since that time direct infusion of drugs into the brain has steadily gained acceptance. Various experimental applications have been translated into clinical trials (2,9) (11). Despite promising results from these recent clinical trials, we still lack a full understanding of the relationship between CED and microanatomy with respect to fluid distribution, along with other issues such as potential immune response in the brain. We have previously established a reflux-free CED technique and real-time imaging Magnetic Resonance (MR) method for monitoring CED delivery of liposomes in primate CNS (8) (13). This allowed us to monitor liposomal distribution in real time in the putamen, corona radiata and brainstem (4). These studies revealed previously unrecognized pathways of perivascular transport along large vessels that

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occurred parallel to interstitial distribution of liposomes during CED (7). Our focus on liposomes was driven by three main considerations: (a) encapsulated Gadoteridol (GDL) allows imaging by MR, (b) liposomes are nano-scale particles that distribute well within CNS, and (c) liposomes allow encapsulation of a broad range of pharmaceutical agents.

Since current studies seek to establish a clinically applicable technique to monitor CED of therapeutics in human brain, we have investigated safety parameters and reproduction of our direct delivery technique. Investigated parameters included whether the dose of infused GDL altered distribution parameters, and the minimum effective dose of GDL that could be used to obtain distribution parameters similar to those previously established. We also studied whether multiple infusions in the same anatomical location alter tissue mechanics, and therefore alter distribution in subsequent infusions. Post-mortem histology was used to investigate possible long-term brain damage from multiple GDL infusions. Histological stains were used to assess glial reaction, interstitial space and inflammatory response in primate brains. Our retrospective study suggests that real-time imaging of GDL has considerable potential to become a standard procedure for monitoring direct delivery of liposomal drugs to CNS.

Materials and Methods

Liposomes

Liposomes that contain MRI contrast agent are composed of a mixture of phospholipid, cholesterol, and a pegylated phospholipid in a molar ratio of 3:2:0.3. The liposomes are neutral with respect to charge and stabilized by the presence of polyethylene glycol chains. The standard MRI contrast reagent, Gadoteridol, a chelated Gadolinium, is introduced into the lipid mixture before they are extruded as liposomes with a diameter of 124 ± 24.4 nm as determined by quasi-elastic light scattering (see (13) for detailed methods). Liposomes, loaded with rhodamine for histological studies, were formulated with the same lipid composition and preparation method as the Gadoteridol-containing liposomes except that the lipids were hydrated directly with 20 mM sulforhodamine B (Sigma) in HEPES-buffered saline (pH 6.5) by 6 successive cycles of rapid freezing and thawing, rather than by ethanol injection. The sulforhodamine B liposomes had a diameter of 115 ± 40.1 nm (used for co-infusion with the Gadoteridol-containing liposomes).

Quantification of liposome-entrapped Gadoteridol by MRI

The concentration of Gadoteridol entrapped in the liposomes was determined from nuclear MR relaxivity measurements. The relationship between the change in the intrinsic relaxation rate imposed by a paramagnetic agent (ΔR), also known as “T1 shortening,” and the concentration of the agent is defined by the equation: $\Delta R = r_1[\text{agent}]$, in which r_1 = relaxivity of the paramagnetic agent and $\Delta R = (1/T1_{\text{observed}} - 1/T1_{\text{intrinsic}})$. As Gadoteridol was encapsulated within the liposome, we corrected for the change in the observed T1 imposed by the lipid by measurement of the T1 of solubilized liposomes with and without Gadoteridol by means of an iterative inversion-recovery MRI sequence on a 2-Tesla Bruker Omega scanner (Bruker Medical, Karlsruhe, Germany). The relaxivity of Gadoteridol had been empirically derived previously on the same system and had a value of $4.07 \text{ mM}^{-1} \text{ sec}^{-1}$. The concentration of the encapsulated Gadoteridol was then calculated with the following equation: $[\text{Gadoteridol}] = [(1/T1_{\text{wGado}}) - (1/T1_{\text{w/oGado}})]/4.07$.

Volume Quantitation from Fluorescent Images

After necropsy, animals' brains were sectioned by cryostat into sequential sections of 25- μm thickness at 100- μm intervals. Fluorescence generated from rhodamine was visualized with an ultra-violet light source, and a charged-coupled device camera with a fixed aperture was used to capture the images. The volume of fluorescent liposome distribution was analyzed on a

Macintosh-based image analysis system (NIH Image 1.62; NIH, Bethesda, MD) as previously described in (4).

Volume Quantification from MR Images

The volume of liposomal distribution within each infused brain region was quantified with BrainLab® software (Heimstetten, Germany). *MR* Images acquired during the infusion procedure were correlated with volume of infusion in each series. BrainLab software reads all data specifications from *MR* images. After the pixel threshold value for liposomal signal is defined, the software calculates the signal above a defined threshold value, and establishes the volume of distribution from primate brain. This allows volume of distribution to be determined at any given time-point and can be reconstructed in a three-dimensional image as previously described (4).

Histology

All primate brain sections were stained with Haematoxylin and Eosin for gross pathology. Myelin was detected with Luxol fast blue (Polysciences, PA). GFAP immunoreactivity was performed on all primate brains (R&D Systems, MN).

Experimental Subjects

The protocol was reviewed and approved by the Institutional Animal Care and Use Committees at the University of California San Francisco (San Francisco, CA). Adult male Cynomolgus monkeys (*Macaca fascicularis*, n=5, 3–5 kg) were housed individually in stainless steel cages. Each animal-room was maintained on a 12-hour light/dark cycle with an ambient temperature of 64 – 84°F. Purina Primate Diet was provided daily in amounts appropriate for the size and age of the animals. Tap-water was freely available to each animal. Prior to assignment to the study, all imported animals underwent at least a 31-day quarantine period as mandated by the Centers for Disease Control and Prevention (Atlanta, GA). Each animal was examined after each procedure for neurological signs, indications of infection or behavioral problems. No complications were recorded during the follow-up period. Mean primate age was 10 years and the period between consecutive infusions ranged from 2 to 4 months.

Liposome Infusion Procedure

Infusion of Gadoteridol/rhodamine-loaded liposomes during real-time magnetic resonance imaging monitoring—Primates received a baseline MRI scan and underwent neurosurgical procedures to position MRI-compatible guide-cannulas in the left putamen. Each customized guide-cannula was cut to a specified length and stereotactically guided to its target through a burr-hole created in the skull. The guide-cannula was secured to the skull with dental acrylic, and the tops of the guide-cannula assemblies were capped with stylet screws for simple access during the infusion procedure. Animals recovered for at least 2 weeks before liposome infusions commenced. Under isoflurane anesthesia, the animal's head was placed in an MRI-compatible stereotactic frame and a baseline MRI scan was performed. Vital signs, such as heart rate and pO₂, were monitored throughout the procedure. Infusions were performed according to previously established CED techniques for non-human primates (7). Briefly, the infusion system consisted of a fused-silica needle cannula that was connected to a loading line (containing liposomes) and an oil-infusion line. A 1-ml syringe (filled with oil), mounted onto a Bee Hive® micro-infusion pump (Bioanalytical Systems, West Lafayette, IN), regulated the flow of fluid through the system. Based on MRI coordinates, the cannula was mounted onto a stereotactic holder, and manually guided to the targeted region of the brain through a guide-cannula previously secured. The length of each infusion cannula was measured to ensure that the distal tip extended approximately 3 to 4 mm beyond the length of the respective guide. This created a stepped design at the tip of the cannula to maximize fluid distribution during CED

procedures. After secure placement of the needle cannula, the animal's head was repositioned in the MRI gantry and CED was initiated while MRI data were continuously acquired. An initial infusion rate of 0.1 $\mu\text{l}/\text{min}$ was applied and increased at 10-minute intervals to 0.2, 0.5, 0.8, 1.0 $\mu\text{l}/\text{min}$ up to a final 5 $\mu\text{l}/\text{min}$. Animals received up to 700 μl total infusion volume per hemisphere during each infusion session. Each animal received a mixture of GDL and rhodamine liposomes; the approximate concentration of liposomes injected corresponded to a formulated concentration of 10 mM phospholipids and 5 mM Gadoteridol. Approximately 15 min after infusion, the cannula withdrawn from the brain. Each animal was infused up to 3 times with at least a 4-week interval between each infusion procedure. Immediately after the last intracranial CED procedure, the animal was euthanized with an overdose of pentobarbital. The brain was then harvested and coronally sectioned into 3- to 6-mm blocks. Each brain slice was immediately frozen in cooled isopentane/dry-ice, and processed for histological analysis.

Results

Distribution of decreasing GDL concentrations in primate CNS

Three different concentrations of GDL (0.925, 1.85 and 3.7 mM Gd) were infused into the primate brainstem, and distribution volume analyzed (Fig. 1). Our initial studies employed 3.7 mM GDL (4), and this concentration was used as the comparative standard for measurement of distribution of lower concentrations (Fig. 1A). As expected, signal intensity on MR declined during infusion of 1.85 mM GDL, but continued to distribute linearly. A further decline in signal was observed at 0.925 mM Gd, but a linear relationship could be still established. The volumetric calculation of infused GDL had to be manually corrected at 0.925 mM Gd, since the defined threshold for GDL signal started to overlap with background signal. Finally, we plotted all concentration data in one graph (Fig. 1B). We conclude that distribution of GDL is unaffected by concentration ($R^2 = 0.96$).

Distribution of multiple infusions in the same anatomical location

Since the experimental subjects were infused up to three times in the same anatomical location, we investigated whether consecutive infusion altered tissue mechanics, and therefore distribution, upon subsequent infusions (Fig 2). The following regions were studied: corona radiata, putamen and brainstem. All three regions showed a linear relationship between volume of infusion (V_i) and volume of distribution (V_d) in consecutive infusions. Graphs were established for each animal used in this study, and two representative graphs from an animal that received largest infusion volume are shown (Fig 2). The first infusion of GDL ranged from 0–300 μl (Nr. 1), the second 0–400 μl (Nr. 2), and the third 0–700 μl (Nr. 3). No significant difference was found between infusions in the same animal and a linear relationship can be established among consecutive infusions in corona radiata ($R^2 = 0.974$) and brainstem ($R^2 = 0.98$). See (5) for detailed analysis of infusion/ distribution curves and V_i/V_d analysis for each anatomic region infused.

Distribution of GDL in primate brainstem

We have previously shown that the brainstem has the highest V_d/V_i ratio, and is an excellent target structure for image-guided CED (5). Here we emphasize the extent of distribution in relation to cannula placement. Cannula tip placement was verified in the upper part of the pons region (Fig 3A). Distribution tended to move in a caudal direction towards medulla oblongata and spinal cord (Fig.3b–h). In addition to the robust caudal distribution, some distribution extended via the superior cerebellar peduncle towards cerebellum (Fig. 3d–f).

Histology and Pathology after CED of Liposomes

Animals euthanized after the third CED procedure underwent histological analysis for gross pathology (Fig 4A). A representative animal was euthanized after receiving 700 μ l of GDL into corona radiata and brainstem. Hematoxylin and Eosin staining (Fig. 4A, upper panel) shows signs of increased intracellular space in the corresponding distribution areas of liposomes as verified with rhodamine-liposomes (bottom panel). As described in previous studies only slight tissue damage is seen at cannula tip (3,11). Brain regions infused with GDL (Fig. 4B, right-hand panels) were compared with non-infused corresponding brain region (Fig. 4B, left-hand panels) of the contralateral hemisphere. Each animal underwent analysis for potential pathologic changes resulting from repeated CED. Representative sections from each of the three infused anatomic regions are shown. Panels a and b show a 10 $\times$ magnification of brainstem stained for myelin to evaluate myelin sheaths. Mild myelin pallor with very slight changes in the normal histological pattern is seen on infused side (b) when compared to non-infused side (a). A diffuse, mild-to-moderate GFAP immunoreactivity and mild astrogliosis were seen in both non-treated sections of basal ganglia (c) and brain stem (e) and infused areas of basal ganglia (d) and brain stem (f). This background GFAP immunoreactivity was consistent with the age of animals admitted for this study (age > 10 years). There is neither evidence of necrosis of inflammatory reaction, nor microgliosis in any of our primate histological examinations.

Discussion

Recent development of a real-time imaging method to monitor liposomal distribution by MRI in primate CNS is a step forward in direct drug delivery to CNS. The use of GDL to monitor the distribution of drug-loaded liposomes allows the infusion of a target structure with considerable confidence, while maintaining the ability to stop the infusion at any point should untoward or excessive distribution occur. Although no overt tissue toxicity of GDL has been found so far, an important part of this study was to define the lowest GDL concentration that would allow imaging while maintaining the linearity of the relationship between volume of distribution (V_d) versus volume of infusion (V_i). No significant difference in distribution was found by lowering the GDL concentration, but lower signal intensity reduced accuracy of automated calculation of volume of distribution. For future clinical applications it may be desirable to rely completely on automated calculations and eliminate possible inter-rater variability. Our study has also shown that there is no change of distribution among repeated infusions in the same anatomic structure. Previous studies have shown that the $V_d:V_i$ relationship is fixed for different animals at a given anatomic locus. These results not only validate the remarkable accuracy of our method but show, moreover, that any future clinical application should be able to predict volume of infusion for a given anatomic structure prior to the initiation of any invasive procedure. Our greatest concern was possible pathological changes in primate brain after repeated CED. In our initial studies we showed that GDL are cleared from primate brain within 72 h after infusion (12;13). This study surveyed animals across various studies in which they received repeated infusions, all animals, irrespective of the infusion locus, showed rapid recovery after each procedure without neurological deficits. As seen on our histology slides minimal parenchymal edema is seen immediately infusion procedure. An acute increase in T2-weighted signal also dissolved with decreasing MR signal in above mentioned previous studies. Further pathological analysis of all primate brain sections did not show any inflammatory or more than expected baseline degenerative changes. In summary real-time imaging of GDL is a reproducible, safe procedure that has a clear clinical potential given the fact that liposomes can encapsulate many kinds of therapeutics in addition to contrast reagents. Of course, any co-infused drug has the potential to cause side-effects; however, sub-optimal delivery can only complicate such assessments, and emphasizes the value of having a much more reliable and predictable delivery procedure on which to rely. Our

studies with Liposomal CPT-11 (10) and Liposomal Doxorubicin (6) have addressed toxicity of liposomal chemotherapeutics in CNS. Visualization of distribution can reduce current complications and infusion can be stopped at the first sign of a side-effect. Another aspect that warrants further careful investigation is CED in pathologically altered brain since the main clinical application of this technology will focus on CNS diseases. In general, one can imagine clinical CED falling into at least two classifications: (a) delivery to pathologically altered but anatomically intact brain (neurodegenerative diseases), and (b) delivery to pathologically altered brain anatomy (CNS tumors). There is also the additional issue of differential composition of normal tissues with respect to the degree of myelination that we have addressed elsewhere (3,7). Although anatomical changes in the brains of those afflicted with neurodegenerative diseases has been well documented, particularly in terms of ventricular enlargement, a signal advantage of image-guided CED is that it offers real-time alterations in delivery of therapeutic agents that personalize the procedure to the idiosyncrasies of the targeted brain region. In contrast, the significant destruction of healthy brain caused by infiltrative brain tumors poses a much trickier problem. Recent experiments have shown that areas of solid tumor are easily amenable to CED but areas of necrosis cause show significant decrease in distribution leading to accumulation of liposomes (6). Regions of tumors not covered by drug results eventually in tumor expansion/relapse. This problem has been addressed by placing multiple infusion cannulas in the targeted tumor area. Intra-tumoral structural variations pose a significant challenge to our approach and are under current investigation. We believe, however, that translation of image-guided CED into clinical use in neuro-oncology and neurodegenerative disease will serve both to expand effective treatment options and will in turn contribute to further refinement of this technology.

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Fig. 1A

Figure 1.

A. Decreasing GDL concentration and MRI signal; top panel: linear correlation of V_i/V_d was found in all three concentrations tested. Manual correction of automatic delineation was needed in the 0.925 mM group; also a decrease in R^2 value is noted in this group. **B.** All concentrations were plotted on one graph and continued to show strong overall linear correlation.

Figure 2.

Repeated infusions into corona radiata (upper) and brainstem (lower) have been analyzed for linear correlation in all animals. Representative animal is shown here, all R^2 -values show strong linear correlation between repeated procedures. (0– 100 μ l data incorporated from previously published data).

Figure 3. Representative distribution in brainstem and corona radiata. Placement of cannula as shown (a), increasing distribution from 0 to 700 μ l infusion (a–h) in both regions.

Figure 4.

A: Representative histology from an animal euthanized immediately after third CED of GDL. H&E staining is indicated in the upper panel, and fluorescence in the lower panel. The left-hand panel shows the distribution in corona radiata infusion; right-hand panels show brainstem distribution. **Figure 4B:** Comparison of treated brain regions (right-hand panels) with the corresponding non-treated brain regions (left-hand panels) on the opposite side show only slight myelin pallor with minimal disruption of the normal histological pattern (a and b) as the only microscopic change in the treated zones. There is a diffuse, mild-to-moderate GFAP immunoreactivity in both non-treated (c and e) and treated (d and f) zones of this background

astrogliosis in the treated zones of either the brainstem (d) or the basal ganglia (f). there is no evidence either of necrosis, inflammatory reaction, nor microgliosis.