Convection-enhanced delivery into the rat brainstem

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Object. Convection-enhanced delivery (CED) can be used safely to achieve high local infusate concentrations within the brain and spinal cord. The use of CED in the brainstem has not been previously reported and may offer an alternative method for treating diffuse pontine gliomas. In the present study the authors tested CED within the rat brainstem to assess its safety and establish distribution parameters.

Methods. Eighteen rats underwent stereotactic cannula placement into the pontine nucleus oralis without subsequent infusions. Twenty rats underwent stereotactic cannula placement followed by infusion of fluorescein isothiocyanate (FITC)–dextran at a constant rate (0.1 μl/minute) until various total volumes of infusion (V_ɪ) were reached: 0.5, 1, 2, and 4 μl. Additional rats underwent FITC–dextran infusion (V_ɪ = 4 μl) and were observed for 48 hours (five animals) or 14 days (five animals). Serial (20-μm thick) brain sections were imaged using confocal microscopy with ultraviolet illumination, and the volume of distribution (V_d) was calculated using computer image analysis. Histological analysis was performed on adjacent sections.

No animal exhibited a postoperative neurological deficit, and there was no histological evidence of tissue disruption. The V_d increased linearly (range 15.4–55.8 mm^3) along with increasing V_ɪ, with statistically significant correlations for all groups that were compared (p < 0.022). The V_d/V_ɪ ratio ranged from 14 to 30.9. The maximum cross-sectional area of fluorescence (range 9.8–20.9 mm^2) and the craniocaudal extent of fluorescence (range 2.8–5.1 mm) increased with increasing V_ɪ.

Conclusions. Convection-enhanced delivery can be safely applied to the rat brainstem with substantial and predictable V_d. This study provides the basis for investigating delivery of various candidate agents for the treatment of diffuse pontine gliomas.

KEY WORDS • brainstem tumor • diffuse pontine glioma • convection-enhanced delivery • local delivery • rat

Convection-enhanced delivery is a method of local delivery in which a pressure gradient, or bulk flow, is used to drive an infusate through the extracellular fluid compartment. In numerous animal studies and in one clinical trial, CED has been demonstrated effectively to achieve high local infusate concentrations in the parenchyma of both the brain and spinal cord. These studies have established the safety of the technique as well as the effects of varying the infusion volume, infusion rate, and other parameters associated with the distribution of an infusate.

The current study is based on the hypothesis that CED may offer an alternative means of treating diffuse pontine gliomas, a subset of brainstem tumors that occurs predominantly in children and carries a dismal prognosis. A multitude of trials using various therapeutic approaches that have yielded no survival benefit demand a novel approach to treatment for this devastating disease. Because the application of CED has never been reported in the brainstem, we used an animal model to assess the safety and define the infusion parameters for CED in this location. Although the results of previous animal studies have demonstrated the safety of CED in the cerebrum and spinal cord based on clinical and histological criteria, the unique architecture and clinical significance of the brainstem demand that CED be investigated separately within this region before any potential therapeutic applications can be considered. This study will serve as the basis for investigating the administration of various candidate agents by CED in future animal studies and, ultimately, in clinical trials.

Materials and Methods

Animal Preparation

Forty-eight male Sprague–Dawley rats, each weighing between 237 and 351 g, were used in these experiments. Anesthesia was induced by an intraperitoneal injection of ketamine (90 mg/kg) mixed with xylazine (4 mg/kg). Supplemental intraperitoneal injections of ketamine and xylazine were administered as needed to maintain a surgical plane of anesthesia throughout the procedures. All animal experimentation was conducted with the approval of the Institutional Animal Care and Use Committee of the Weill Medical College of Cornell University (Protocol No. 0008-744A) and in accordance with the National Institutes of Health guidelines for the use of animals in research.

Experimental Groups

To establish the safety of cannula insertion, 18 animals underwent a stereotactic procedure in which the cannula was placed into the PnO without subsequent infusions. The remaining 30 animals were

Abbreviations used in this paper: BBB = blood–brain barrier; CED = convection-enhanced delivery; FITC = fluorescein isothiocyanate; PnO = pontine nucleus oralis; V_d = volume of distribution; V_ɪ = volume of infusion.
divided into six experimental groups consisting of five animals each. In rats in Groups 1 through 4 the PnO was infused with FITC–dextran to various total Vis: 0.5, 1, 2, and 4 μl, respectively. The FITC–dextran compound was chosen as the infusate based on previous studies in which the stability of this conjugate and its negligible binding to tissue components had been demonstrated. This compound has previously been used to assess distribution of infusates into brain parenchyma. To identify infusion-induced delayed clinical deterioration or histological damage, animals in Groups 5 and 6 underwent infusions of FITC–dextran (V, 4 μl) and were then observed for 48 hours and 14 days, respectively, before they were killed.

**Infusion Apparatus and Infusate**

Infusion cannulas were prepared by inserting 33-gauge internal cannulas into 26-gauge guide cannulas with a 1.5-mm projection of the internal cannula tip. The space between the distal end of the guide cannula and the projecting portion of the infusion cannula was sealed by applying methyl-2-cyanoacrylate to prevent backflow of infusate into the space between the guide cannula and the internal cannula.

The sealed guide cannula and the internal cannula were attached to the connector assembly, which consisted of a polyethylene supply tube encased in tough vinyl tubing. The entire length of the tubing was flushed with FITC–dextran (molecular weight 20,000 g/Mol), which was diluted in sterile isotonic saline (100 mg FITC–dextran/ml saline), and the free end of the tubing was attached to a 10-μl syringe that also contained FITC–dextran. Care was taken to eliminate air bubbles from the syringe and the connector assembly by repeated aspiration and flushing.

All infusions of FITC–dextran were administered at a constant rate of 0.1 μl/minute via a syringe pump attached to the connector assembly. The duration of the infusions ranged from 5 to 40 minutes, depending on the total V assigned to the experimental group.

**FIG. 1.** Coronal (upper) and sagittal (lower) diagrams of the rat brainstem. The target within the pontine tegmentum, the PnO, is indicated by the shaded oval. Modified with permission from the publisher (originally Figs. 48 and 80) of Paxinos G, Watson C: The Rat Brain in Stereotaxic Coordinates, ed 2. Sydney: Academic Press, 1986.
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Animal Procedures

After a surgical plane of anesthesia had been established, the rat's head was shaved with clippers and secured in a stereotactic frame. The animal's eyes were then lubricated with sterile petrolatum-based ocular lubricant. A No. 11 scalpel blade was used to make a scalp incision extending from the frontal region just anterior to the bregma to approximately 2 cm caudal to the medial edge of the lambdoid suture. The pericranium was dissected laterally from the skull using a cotton-tipped applicator, and a small self-retaining retractor was placed.

The rat's head position was adjusted within the stereotactic frame to facilitate the appropriate trajectory for cannula insertion into the target site within the pontine tegmentum, the PnO. The PnO was selected as the target site because of its relatively large size (9 mm³), the predominance of surrounding white matter, and the absence of cranial nerve nuclei or major ascending and descending fiber tracts (Fig. 1). To obtain the appropriate trajectory for this target, the anterior portion of the animal's head was elevated 3 mm above a horizontal plane, and the skull target (located 1.4 mm to the right of the sagittal suture and 1 mm anterior to the lambdoid suture) was identified. A hand-held drill was used to create a small burr hole in this location, and a 25-gauge needle was used to puncture the dura mater gently. Burr hole site bleeding was controlled by applying gentle pressure with a cotton-tipped applicator. The infusion cannula apparatus was secured in the clamping device of the stereotactic frame and inserted to its target depth in the brain (7 mm below the dura).

In rats receiving infusions, cannulas were removed 5 minutes following completion of the infusion. Burr hole site bleeding was controlled by inserting bone wax, and the scalp was sutured closed in one layer by using a No. 3-0 nylon suture. The animal was then removed from the stereotactic frame and allowed to emerge from anesthesia.

Clinical Assessment

Postoperative neurological assessment primarily included observation of integrated sensorimotor task-specific activities, including gait and responses to stimuli such as visual and auditory threat. Gait was observed, with particular attention paid to any evidence of asymmetry in fore- and hindlimb movement. Sensory examination consisted of assessing the animal's eyes and tail in response to a tactile stimulus. A limited examination of cranial nerve function was performed. This included assessment of the corneal reflex (by gentle stimulation of the cornea with a cotton-tipped applicator) and observation of mouth movement.

In rats in which cannulas were placed without subsequent infusion and in those assigned to Groups 1 through 4, the animals were killed after postoperative assessment (range 2–5 hours following infusion). Animals in Groups 5 and 6 were observed for 48 hours and 14 days, respectively, and were killed after undergoing repeated clinical assessments.

Tissue Preparation

The rats were killed by an intraperitoneal injection of 1.5 ml of euthanasia solution. Their brains were immediately harvested, and a razor blade was used to remove excess cranial and causal neural parenchyma. The remaining brain block was then coated with a thin layer of embedding medium and flash frozen in chilled isopentane. The brains were mounted to enable coronal sectioning (20 μm/section), which was performed using a microtome cryostat that was chilled to ~25°C. Every fifth section of brain was mounted onto a slide for analysis of infusate distribution, and each adjacent section was mounted onto a slide for histological analysis.

Histological Analysis

Histological analysis of serial brain sections was performed by a neuropathologist blinded to the experimental group to which each animal belonged. Sections were stained with hematoxylin and eosin, after which they were analyzed for evidence of hematoma, edema, necrosis, disruption of cytoarchitecture or fiber tracts, ischemic damage (red neuronal change and neuropil vacuolization), and disruption of white matter.
was not performed in one animal in Group 6 because of technical difficulties during microtome sectioning.

In all 47 animals, disruption of brain tissue was observed only at the immediate site of the cannula tract. Despite extensive distribution of infusate within the pons and medulla, as demonstrated by fluorescence image analysis (see Discussion and figures), no evidence of hemorrhage, edema, necrosis, disruption of cytoarchitecture or fiber tracts, ischemic damage (red neuronal change and neuropil vacuolization), or disruption of white matter was observed 10 to 20 μm beyond the cannula tract. Aside from the immediate region of the cannula tract, the histological appearance of the brainstem on the side of the infusion had no identifiable abnormalities and was identical to that of the noncannulated side.

A neuropathologist blinded to the group to which each rat belonged did not discern any distinguishing feature that could identify which animals received higher compared with lower infusion volumes. Brain sections excised from animals in Groups 1 through 4 demonstrated small foci of hemorrhage at the cannula tract site (Fig. 2a and d). Other features observed in some animals in the immediate region

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**Fig. 2.** Hematoxylin and eosin–stained sections (slice thickness 20 μm) through the pons following infusion of FITC–dextran. a–c: Low-power views of sections through the pons excised from animals in Group 2 (a), Group 5 (b), and Group 6 (c). The arrow in each image indicates a punctate hemorrhage at the site of the cannula tract. Note the absence of other anatomical abnormalities. Original magnification × 1. d: Photomicrograph showing a high-power view of the area of punctate hemorrhage noted by the arrow in panel a. A focus of hemorrhage with no associated astrocytic or inflammatory reaction is observed. The surrounding parenchyma has no anatomical abnormalities. Original magnification × 200. e: Photomicrograph showing a high-power view of the area noted by the arrow in panel c. A collection of hemosiderin-laden macrophages with little reactive astrocytosis is observed in the sharply delineated area of the cannula tract. The surrounding parenchyma has no identifiable abnormalities. Original magnification × 200.
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Results of the Infusate Distribution Analysis

Fluorescence image analysis was performed in rats in Groups 1 through 4 (Fig. 3). Although extensive fluorescence was observed in brain sections excised in rats in Groups 5 and 6, accurate image analysis was not possible due to considerable clumping of fluorescent material.

The results of the distribution analysis of Groups 1 through 4 are summarized in Table 1. The $V_d$ increased linearly (range 15.4–55.8 mm$^3$) along with the increasing $V_i$ (Fig. 4), with statistically significant correlations for all groups that were compared ($p < 0.022$). The $V_d/V_i$ ratio ranged from 14 to 30.9, with intergroup comparisons not reaching statistical significance ($p < 0.13$). The cross-sectional area of fluorescence (range 9.8–20.9 mm$^2$) and the spread of fluorescence in the craniocaudal direction (range 2.8–5.1 mm) increased with increasing $V_i$. Intergroup comparisons did not reach statistical significance for either of these measurements ($p < 0.39$ and $p < 0.15$, respectively). Although the extensive spread of fluorescence was noted both in the craniocaudal direction and in the cross-sectional axis, minimal or no infusate was detected on the contralateral side of the brainstem in all animals.

Discussion

Background and Previous Studies

Diffuse pontine gliomas, a subset of brainstem tumors, account for 10 to 15% of all brain tumors in children and are associated with a dismal prognosis. These tumors are not amenable to resection, respond only transiently to radiotherapy, and are refractory to all current chemotherapy regimens. Many features of diffuse pontine gliomas that have rendered current therapies universally unsuccessful are appealing for effective CED, a local delivery method that bypasses the BBB and achieves high local drug concentrations with minimal systemic exposure. Diffuse pontine gliomas are relatively compact and do not typically metastasize, characteristics that are ideal for effective local delivery strategies. Moreover, these tumors are confined primarily to white matter, and CED has been shown to follow isotropic white matter fibers. Finally, diffuse pontine gliomas maintain a relatively intact BBB and blood–tumor barrier, and CED results in a negligible efflux in the presence of an intact BBB.

Thus, therapeutic infusions administered by CED for diffuse pontine gliomas would be expected to distribute readily throughout the tumor interstitium, achieve high regional concentrations, and result in little or no systemic exposure.

An abundance of clinical and histological evidence supports the safety of using CED in the cerebrum of rats, cats, primates, and humans. In addition, CED infusion into the parenchyma of the spinal cord has been reported in rats, swine, and primates. Although the authors of previous studies have demonstrated that CED can achieve high local drug concentrations without causing significant
systemic exposure or recognized toxicity, there is no previous publication in which the feasibility of using CED within the brainstem has been demonstrated. The unique architecture and clinical significance of the brainstem demand that CED be investigated separately within this region before any potential therapeutic applications can be considered.

In these experiments, we intentionally selected healthy rats rather than a tumor model for several major reasons. First, no currently available tumor model simulates the unique features of the diffuse pontine glioma, especially its infiltrative nature and its expansion along white matter tracts. Thus, although distribution parameters in the healthy brainstem may differ significantly from those in an infiltrating tumor, there is no current tumor model with which to address this discrepancy. Moreover, our use of healthy animals best addresses one of our primary objectives—to establish that infusions by CED can be safely received without damaging healthy brainstem structures.

Safety of CED in the Pons

In this study we demonstrate that a small cannula can be stereotactically inserted into the pontine segment of the rat brainstem and an inert infusate can be safely introduced into this target by CED. We recognize the obvious limitations of neurological examination in the rat; nonetheless, we found no evidence of any clinical change from normal, preoperative baseline status in any rat. Moreover, histological examination demonstrated no evidence of tissue disruption outside the small area surrounding the cannula tract. In this study all infusions were performed at a low constant rate (0.1 µl/minute) based on previous studies, which have demonstrated that higher infusion rates are associated with greater backward leakage and lower recovery rate of infusate. We emphasize that the current experiments demonstrate the safety of infusions by CED into the brainstem at a low infusion rate and that higher infusion rates may be associated with morbidity. Moreover, in these experiments only one-time infusions were performed, and thus this study does not address potential morbidity that may be associated with chronic infusions.

We propose that our having established safety in the extremely small brainstem of the rat, in which the ratio of the cannula size to brainstem diameter is much higher than that found in humans, lends credence to the hypothesis that CED can be performed safely in the larger human brainstem as well. The safety of cannula insertion into the human brainstem has already been demonstrated in clinical reports of stereotactic needle biopsy procedures in which extremely low morbidity rates were cited. The current report, in which we demonstrate the safety of CED of an inert substance into the rat brainstem, provides the basis for investigating the safety of infusing antineoplastic agents into the brainstem of animals and, eventually, that of humans.

Infusate Distribution Analysis

The finding that, in cases of CED of infusate into the brainstem, \( V_d \) increases linearly along with the increasing \( V_i \) is consistent with previous findings reported by investigators who used CED in other areas of the central nervous system. Although not statistically significant for comparisons among all groups, the cross-sectional area of fluorescence and the craniocaudal spread of fluorescence also increased along with the increasing \( V_i \). These findings, as well as the pattern of distribution (for example, lack of infusate spread across the midline), will have important implications for the design of therapeutic trials of CED in the human brainstem.

Authors of previous studies have reported a \( V_d/V_i \) ratio ranging from 1.3 to 5.2 and have attributed this variation to factors including backward leakage around the inserted cannula, site of perfusion (white or gray matter), and the degree of anisotropy of white matter fiber tracts. The \( V_d/V_i \) ratio reported in the current study for infusions into the brainstem (range 14–30.9) is significantly higher than those that were previously reported for CED in other areas of the central nervous system. The most likely reason for this discrepancy is that measurement of \( V_i \) has been performed by various means in different studies, including quantitative autoradiography performed using \(^{14}\text{C}-\text{albumin}\), \(^{14}\text{C}-\text{sucrose}\), iron staining involving monocristalline iron nanoparticles, and ultraviolet illumination accomplished using FITC–dextran. In addition to methodological differences in \( V_i \) measurement, other factors may also have contributed to the high \( V_d/V_i \) ratio demonstrated in the current study. These include the selection of a target site far removed from the brain surface, the use of a cannula that was sealed to eliminate backflow and efflux, and the compact array of fibers in the brainstem.

One limitation encountered while performing distribution analysis with a fluorescent-labeled infusate such as FITC–dextran is the inability to quantify infusate concentration gradients in brain sections accurately. Nevertheless, quantification of infusate concentration was not considered relevant to the current experiments, in which the primary goals were to establish the safety of the technique and to determine the extent of distribution of an inert infusate in the brainstem. Quantification of infusate analysis will be necessary for subsequent experiments designed to test safety and distribution of antineoplastic agents infused by CED into the brainstem.

Conclusions

The results of this study demonstrate that CED can be safely applied in the rat brainstem with substantial and predictable \( V_d \). These experiments provide the basis for inves-
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tigating the CED of various candidate antineoplastic agents into the brainstem. If proven to be safe in further animal studies, CED may eventually offer a viable alternative for the treatment of diffuse pontine gliomas.

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References


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