CHAPTER 3
Convection-enhanced Delivery of Carmustine to the Murine Brainstem: a feasibility study

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ABSTRACT

Background
Systemic delivery of therapeutic agents remains ineffective against diffuse intrinsic pontine glioma (DIPG), possibly due to an intact blood-brain-barrier (BBB) and to dose-limiting toxicity of systemic chemotherapeutic agents. Convection-enhanced delivery (CED) into the brainstem may provide an effective local delivery alternative for DIPG patients.

New Method
The aim of this study is to develop a method to perform CED into the murine brainstem and to test this method using the chemotherapeutic agent carmustine (BiCNU). To this end, a newly designed murine CED catheter was tested in vitro and in vivo. After determination of safety and distribution, mice bearing VU-DIPG-3 and Eg8FM-DIPG brainstem tumors were treated with carmustine dissolved in DW 5% or carmustine dissolved in 10% ethanol.

Results
Our results show that CED into the murine brainstem is feasible and well tolerated by mice with and without brainstem tumors. CED of carmustine dissolved in 5% DW increased median survival of mice with VU-DIPG-3 and Eg8FM-DIPG tumors with 35% and 25% respectively. Dissolving carmustine in 10% ethanol further improved survival to 45% in mice with Eg8FM-DIPG tumors.

Comparison with existing methods
Since genetically engineered and primary DIPG models are currently only available in mice, murine CED studies have clear advantages over CED studies in other animals.

Conclusion
CED in the murine brainstem can be performed safely, is well tolerated and can be used to study efficacy of chemotherapeutic agents orthotopically. These results set the foundation for more CED studies in murine DIPG models.
INTRODUCTION

Diffuse intrinsic pontine glioma (DIPG) is a fatal brain malignancy in children, for which prognosis has not improved in the last 40 years 88,89. Although recent in vitro studies have shown DIPG cells to be sensitive to both classic chemotherapeutic drugs and novel targeted agents, 48 multiple clinical trials have so far been unsuccessful 89. A possible reason for this failure is the inability of therapeutic agents to reach tumor cells, due to a relatively intact blood-brain-barrier (BBB) 131,132. The BBB constitutes a physiological barrier to safeguard the central nervous system from exposure to both endogenous and exogenous toxins, thereby also preventing effective delivery of chemotherapy to the tumor parenchyma 133. Therefore, convection-enhanced delivery (CED), a local drug delivery method, may be a promising delivery approach to more effectively treat DIPG patients 134–136. CED relies on a continuous hydrostatic pressure gradient, which propels therapeutic agents over relevant anatomical volumes, at a speed several orders of magnitude greater than simple diffusion 137. Local drug concentrations achieved by CED can be up to 10,000-fold higher as compared to intravenous drug administration, while minimizing systemic exposure 138. Because of a wider distribution, CED could be preferred over intrathecal-, intraventricular- and intra-arterial drug administration or polymer-wafer implantation in brain tumor patients 139–141. The use of CED as a treatment strategy for DIPG patients has matured from preclinical studies showing feasibility in rats 142,143, to safety and distribution studies in non-human primates 144,145 and phase I/II clinical trials in children 146. To our current knowledge, four studies have been published, describing follow up of five pediatric patients treated with CED in the brainstem, four of which were suffering from a DIPG 69–72. These studies have shown CED in DIPG to be feasible and safe but have yet to show a survival benefit. To further improve CED for more (pre-clinical) research is needed. Even though the safety and efficacy of CED in the brainstem has been assessed in rat non-tumor 143 and non-DIPG brainstem tumor models in rats 142, no study has shown the feasibility of CED in the murine brainstem. Since true primary DIPG-xenograft and genetically engineered models of DIPG are currently only available in mice 31,63, we decided to conduct a safety and efficacy study of CED in the murine brainstem using the chemotherapeutic agent carmustine (BiCNU, 1,3-bis-(chloroethyl)-1-nitrosourea).

Carmustine is an alkylating agent with a clear differential toxicity to pediatric high grade glioma and DIPG cells in vitro compared to astrocytes 46. Dose limiting systemic
toxicity makes carmustine unsuitable for intravenous therapy in brain tumor patients 
but its efficacy against glioma cells and the lack of toxicity to astrocytes at clinically 
relevant concentrations makes it an excellent candidate for local therapy. Currently, 
carmustine is the only FDA-approved treatment for intracerebral chemotherapy of adult 
glioblastoma. In addition, interstitial carmustine administration by wafers has shown 
to be safe in pediatric brain tumor patients.

In this study we evaluated carmustine dissolved in 5% dextrose (DW) as known to be safe 
in rats. Subsequently, we tested the safety of 10% ethanol administration to the murine 
brainstem, because dissolving carmustine in 10% ethanol could improve distribution 
and allows for a better translation into the clinic. As a final test we studied the in vivo 
efficacy of local delivery of carmustine via CED dissolved in both vehicles (5% DW and 
10% ethanol). For this purpose we employed our recently developed VU-DIPG-3 model and our previously established E98-Fluc-mCherry (E98FM) DIPG model.

MATERIALS AND METHODS

Carmustine
For the in vivo experiments carmustine (BiCNU), (Bristol-Myers-Squibb, Princeton, 
NJ), was resuspended to a concentration of 3.3 mg/ml in either 5% DW (pH<4), or 10% 
ethanol. Carmustine dose was measured in vitro before and after the CED procedure 
using HPLC-UV in both vehicles.

Convection-enhanced delivery in vitro
The CED-system was tested in vitro by performing CED of trypan blue in 0.6% agarose 
gel, which has been previously described as a reliable model to simulate CED in the 
brain parenchyma. CED was performed at a speed of 0.5 µl/minute for 30 minutes. 
Total infusion volume was 15 µl. Five minutes after the end of the procedure, the inner 
cannula was withdrawn and after one minute the guide was also withdrawn at a speed 
of 1 mm/minute. Distribution and backflow were assessed by observing trypan blue 
distribution in the agarose gel.

Animals used for convection-enhanced delivery experiments
Animal experiments were performed in accordance with the Dutch law on animal 
experimentation and the protocol was approved by the committee on animal
experimentation of the VU University Medical Center (VUMC). All tumor models (E98FM-DIPG, total n=31, VU-DIPG 3, total n=12) were established in immune deficient 6-week-old athymic nude-foxn1<sup>nu</sup> mice to allow for adequate engraftment. Toxicity studies of 10% ethanol infusion (total n=15) were performed on 6-week-old balb/c mice with an intact immune system to study the full scope of possible tissue reactions. All mice were housed under specific pathogen-free conditions in a 12-hour light-dark cycle and were offered food and water ad libitum. Weights were measured and clinical scores were assigned daily after the CED procedure (toxicity and efficacy studies). Clinical scores ranged from 0 to 4 and referred to: 0: normal active behavior, 1: subtle inactivity or subtle neurological symptoms, 2: mild to moderate inactivity or neurological symptoms, 3: severe neurological symptoms, inactivity, loss of reflexes, inadequate grooming, 4: dead. Half point scores were assigned to mice that were behaving in between two scores.

**Orthotopic DIPG mouse models**

A single cell suspension was prepared after harvesting subcutaneous E98FM and VU-DIPG-3 tumors. E98FM cells were originally isolated from an adult GBM patient<sup>50</sup> (135) and the genotypic murine-DIPG model: VU-DIPG-3 was established after injection of DIPG material collected during autopsy<sup>152</sup> 154. E98FM cells and VU-DIPG 3 cells were injected as previously described<sup>50</sup>. Coordinates from lambda for tumor-cell injections were: -1.0 mm X, -0.8 mm Y, 4.5 mm Z. Coordinates were based on “The mouse brain in stereotaxic coordinates” by Franklin and Paxinos, 3rd edition<sup>155</sup>. For the DIPG-E98FM model, tumor growth was monitored twice weekly by bioluminescence imaging (BLI), as previously reported<sup>50</sup>.

**Convection-enhanced delivery in vivo**

Before the CED procedure, animals were injected subcutaneously with the analgesic buprenorphine 0.05-1 mg/kg and then anesthetized with isoflurane 2.5-3% in 100% oxygen. After placing the animals in a stereotactic frame on a heated platform, the CED catheter was introduced into the pons (Coordinates from lambda: -1.0 mm X, -0.8 mm Y, 4.5 mm Z) and the drug was infused (15 µl in 30 minutes). After the procedure the catheter was removed and the animals were returned to their cages to recover and resumed normal active behavior within 3-12 hours.
Convection-enhanced delivery efficacy studies

At day five (VU-DIPG-3) or day seven to nine, depending on bioluminescence signal in E98FM, after intracranial injection of tumor cells for the establishment of orthotopic pontine tumors, mice were stratified on the basis of BLI signal intensities into different treatment groups (E98FM) or randomly assigned to one of the two groups (VU-DIPG-3). Animals were assigned to receive CED with carmustine in DW 5% (VU-DIPG-3 and E98FM-DIPG) or 10% ethanol (E98FM-DIPG) or only DW 5% or 10% ethanol vehicle. Researchers were blinded as to which treatment group mice belonged to. Follow-up included daily observations and weight measurement and measurement of BLI signal twice a week (E98FM). Clinical scores were assigned ranging from 0 (normal active behavior) to 4 (dead). Endpoints were defined as weight loss more than 15%, severe neurological symptoms or severe inactivity. Mice were sacrificed via pentobarbital overdose. Brains were removed and fixed in 3.7% formaldehyde solution in PBS (phosphate buffered saline). Blood was withdrawn from the tail vein before CED and a week thereafter, allowing the monitoring of alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT) and creatinine levels using standard laboratory techniques used at the VUMC.

Tissue staining and histological scoring of mice receiving convection-enhanced delivery with 10% ethanol

Haematoxylin and eosin (H&E) staining was performed on 5 μm formalin-fixed, paraffin-embedded tissue sections cut in the coronal plane from non-tumor baring balb/c mice 2 or 21 days after CED with 10% ethanol or distilled water, using a standard H&E protocol. Sections were selected at the site of the needle tract and 100 μm both rostrally and posteriorly. Two researchers and an independent neuropathologist performed assessment of tissue damage and inflammation. They were blinded to the experimental procedure that the animals underwent.

Statistics:

Differences in survival were analyzed by Kaplan-Meier curves and logrank tests for significance in the efficacy studies. Non-parametric Kruskal-Wallis test followed by a Dunn’s post-hoc test was used to determine differences in ALAT, ASAT and creatinine levels and differences in BLI signal. The weight loss of mice treated with 10% ethanol was analyzed using a generalized linear model (GLM) and carmustine dose measured before and after CED in 10% ethanol and 5% DW was analyzed using a two-tailed t-test. A p < 0.05 was considered statistically significant.
RESULTS

Convection-enhanced delivery in vitro provides a symmetrical distribution with minimal backflow

A stepped design catheter was developed incorporating an inner cannula (Hamilton Comp., Reno, NV) into an outer needle (Fig. 1a,b). The use of a stepped design reduces the risk of backflow or mechanical tissue injury and allows for a higher infusion rate \(^{70}\) (Fig. 1a,b). The cannula has an outer and inner diameter of 0.24 and 0.11 mm, respectively; the outer guide has an outer and inner diameter of 0.52 and 0.26 mm, respectively. This catheter was connected via a tubing system (Fig. 1c) to a 300 µl disposable syringe inserted into a syringe pump (PHD 2000, Harvard Apparatus Inc., Holliston, MA). A pressure transducer was put in place to assure continuous pressure during the infusion and prompt detection of any issues in delivery (Fig. 1c). The inner cannula was inserted 2.5 mm into the agarose and further lowered to 4.5 mm using a dedicated screw system to ensure the inner cannula followed a straight line minimizing backflow and damage to the brainstem (Fig. 1a). The CED catheter was tested in vitro by performing CED of trypan blue in 0.6% agarose gel. Rate of infusion (0.5 µl/min) and total volume (15 µl) were chosen based on previous pre-clinical studies \(^{74}\), clinical relevance (size of pons in relation to volume infused), and presumed feasibility (supplemental table 1). Only minor leakage could be detected due to needle- and guide withdrawal and trypan blue was distributed as a symmetrical sphere in the agarose gel (Fig. 1d).

Convection-enhanced delivery into the murine brainstem is feasible and safe

Next, using the same settings, CED of trypan blue was performed in vivo to confirm proper infusate delivery. Mice were sacrificed after surgery to confirm infusion at the correct anatomical location (Fig. 1e.f). Trypan blue could be identified in the brainstem area (Fig. 1 e, f). To assess safety of CED with carmustine in the brainstem, vehicle solution (5% DW) or carmustine (3.3 mg/kg in 5% DW) was infused in balb/c mice using the same settings as previously described. Mice recovered promptly from the surgery and no acute or chronic toxicities were observed during a two months follow-up.
10% ethanol can be safely administered to the brainstem via convection-enhanced delivery

To exclude toxicity of 10% ethanol infused directly in the brainstem by CED, non-tumor bearing mice were treated with 15 µl of 10% ethanol or vehicle. One vehicle-
CED of carmustine to the murine brainstem

A treated mouse died during the procedure due to a technical problem (inaccurately fixed catheter). Both 10% ethanol and vehicle-treated mice showed a transient clinical deterioration and increase in weight loss after the CED procedure. All mice recovered completely and no significant difference could be detected in clinical score or weight loss between treatment groups (Fig. 2a, b and Table 1, supplemental figure 1). Histological evaluation of H&E stained brain sections from three separate locations around the needle tract showed substantial early tissue damage at day two (Fig. 2c, and Table 1) and more subtle late changes at day 21 (Fig. 2d and Table 1). There were no differences between 10% ethanol and vehicle-treated brain tissues.

Convection-enhanced delivery with carmustine is effective in pre-clinical models of DIPG

To test the efficacy of carmustine delivered via CED in vivo, carmustine, dissolved in 5% DW, was tested in a primary DIPG mouse model (VU-DIPG 3). CED of carmustine resulted in a modest but significant increase in median survival of 35% (p<0.01) (Fig. 3a). No changes were observed in ASAT, ALAT and creatinine levels after CED of carmustine (Fig. 3b, c, d), indicating no acute liver or kidney toxicities due to systemic exposure.
Table 1 | Histological findings 2 or 21 days after CED with 10% ethanol or vehicle

<table>
<thead>
<tr>
<th></th>
<th>Acute (day 2 after CED)</th>
<th>Chronic (day 21 after CED)</th>
</tr>
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<tbody>
<tr>
<td>Ethanol 10%</td>
<td>Necrosis, oedema and loss of tissue around the needle tract (N=4)</td>
<td>Inflammation (macrophages), glial scaring, some loss of tissue around needle tract (N=4)</td>
</tr>
<tr>
<td>Vehicle</td>
<td>Necrosis, oedema and loss of tissue around the needle tract (N=3)</td>
<td>Inflammation (macrophages), glial scaring, some loss of tissue around needle tract (N=4)</td>
</tr>
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Dissolving carmustine in 10% ethanol increases its efficacy in vivo

To enable translation to the clinic, we investigated whether this significantly increased survival could be further improved by dissolving carmustine in 10% ethanol and used the well-established E98FM-DIPG orthotopic mouse model. Accordingly, we injected E98FM cells into the murine pons as previously described. Some mice experienced transient clinical symptoms after injection of E98FM cells, but no differences were detected between groups (Fig. 3e, f, g, h). At day seven to nine, depending on BLI signal, mice bearing E98FM tumors were treated with either carmustine dissolved in 5% DW or vehicle (5% DW) or carmustine dissolved in 10% ethanol or vehicle (10% ethanol). It should be noted that the differences in BLI between the groups were relatively large, despite not being statistically significant (Table 2).

Table 2 | Median BLI of E98FM-DIPG at start CED

<table>
<thead>
<tr>
<th></th>
<th>Median BLI at start CED</th>
<th>Days after IC-injection</th>
<th>Total N</th>
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<tbody>
<tr>
<td>Ethanol 10%</td>
<td>1.10E+06</td>
<td>7 - 8</td>
<td>8</td>
</tr>
<tr>
<td>Dextrose 5%</td>
<td>6.59E+04</td>
<td>7 - 9</td>
<td>7</td>
</tr>
<tr>
<td>CAR in Ethanol 10%</td>
<td>5.91E+05</td>
<td>7 - 8</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>9.44E+04</td>
<td>7 - 9</td>
<td>8</td>
</tr>
</tbody>
</table>

Due to the CED procedure, clinical scores rose transiently in some animals (Fig. 3e, f, g, h) and in vehicle treated groups, mice rapidly deteriorated due to tumor growth, starting at day 14 (Fig. 3e and f). Mice treated with CED of carmustine dissolved in ethanol demonstrated a significant decrease in tumor growth two weeks after CED, measured by bioluminescent imaging (p<0.05) (Fig. 3i,j). CED of carmustine dissolved in 5% DW significantly increased median survival time with 25% compared to CED of 5% DW only (p<0.01) (Fig. 3k). In comparison, CED of carmustine dissolved in 10% ethanol increased median survival time by 45% (p<0.01) and resulted in one animal without any clinical symptoms or measurable BLI signal after 60 days of follow up (Fig. 3k). Dissolving
CED of carmustine to the murine brainstem

Carmustine in 10% ethanol instead of DW 5% increased survival by 36% (p<0.05). CED with 10% ethanol vehicle itself did not increase survival significantly compared to CED with 5% DW (p=0.15). HPLC-UV measurements before and after CED in both vehicles showed availability of carmustine to be 30% higher when using 10% ethanol compared to 5% DW as vehicle (p<0.05). Carmustine concentration was similar when measured after the CED procedure, suggesting chemical stability in both vehicles (supplemental figure 2).
In this study we show for the first time that CED in the mouse brainstem is reproducible, safe and has potential to show a significant survival benefit. The methods and results presented here set the foundation for future preclinical studies in orthotopic DIPG mouse models, including efficacy and distribution studies using different therapeutic agents and drug carrier systems such as liposomes. As stated earlier, no significant improvement in survival has been achieved for children affected by DIPG in the last 40 years. The permanence of an intact BBB could in part explain DIPG resistance to therapeutic agents delivered systemically. Whether potential chemotherapeutic agents actually reach the tumor after systemic delivery is largely unknown and should be further studied using advanced molecular imaging techniques such as positron emission tomography (PET) scans or experimental systems. When therapeutic levels of agents, shown to be effective in vitro, can not be established in the tumor due to the effects of the BBB or dose limiting toxicity, CED may overcome these issues. Therefore, CED is anticipated to play an important role in future potentially successful treatment regimens, intensifying the need for in vivo techniques to study CED in the brainstem. Currently, clinical CED is performed in phase I/II clinical trials in children with DIPG. Lonser et al. treated a four-year-old DIPG patient with fusion protein interleukin 13 – pseudomonas exotoxin (IL-13-PE) (volume 1.4 ml in 6 hours with infusion rate 0.5 – 10 µl/min) at time of progression. The patient showed only transient worsening of symptoms and died four month after infusion. Anderson et al. treated two children age five and six years with topotecan (volume 6.04 or 5.3 ml in +/-100 hours, infusion rate between 0.3 – 2 µl/min). Both children did show only transient worsening of neurological symptoms after the CED procedure. Follow-up MRI showed a slight decrease of tumor size but the patients did not experience prolonged survival. Barua et al. reported treating a 5-year-old DIPG patient with CED of carboplatin (volume 9 ml in 20 hours, five times in nine days, with infusion rate 0.5 – 10 µl/min) at tumor progression. One month after treatment the patient showed mild clinical improvement and MRI changes that could be attributed to tumor necrosis. The patient died two month after CED from tumor progression outside the field of infusion. Even though clear progress has been made in demonstrating the feasibility of CED in DIPG patients, many questions remain that could be answered by in vivo studies. Although our preclinical CED setup was designed to mimic the clinical situation as accurately as possible, infusion time varies significantly between clinical CED and preclinical CED as presented here. This difference is caused...
by limitations in the acceptable duration of anesthesia in mice. Implanting flexible catheters that remain in the brain parenchyma of the mice would allow for continuous administration without anesthesia. This infusion technique has been performed in the rat brainstem 157, but due to the size of the murine brainstem this will be technically challenging. It could be argued that the small size of the murine brainstem also limits studying the benefits of convection in CED, because small molecules can, in time, diffuse over relative anatomical distances in this model. However since the biological half life of carmustine is relatively short, our method still poses an unique opportunity to study the efficacy of drugs such as carmustine in vivo orthotopically.

We show CED with carmustine using 5% DW as a vehicle to be effective in a primary model of DIPG, however carmustine dissolved in ethanol 10% proved significantly more effective. Adequate distribution of a therapeutic agent is key to the efficacy of locally delivered therapy. Chemical structure (large molecules) and solvability (lipophilicity) of the infused compound can negatively influence volume of distribution in tissue after CED 74. Carmustine is a lipophilic drug that, for intravenous use and for local delivery, including into brain tumors, has long been dissolved in 100% ethanol to improve delivery 158. Using 100% ethanol allowed for safe intra-tumoral injection up to a carmustine concentration of 60 mg/ml in GBM patients, although adverse events of 100% ethanol were reported when used for intra-arterial delivery 151,158. Currently it is thought that a minimum of 10% ethanol or 5% DW solution with pH<4, can be used as a vehicle for carmustine, up to a maximum concentration of 3.3 mg/ml 151. Both vehicles will maintain the stability of the drug, however the safety and efficacy of CED with 10% ethanol in the brainstem is unknown. It has been noted that carmustine in 5% DW remains a suspension, possibly influencing the distribution of the compound and thereby its efficacy. In this study, efficacy significantly improved upon dissolving carmustine in 10% ethanol. This improvement is likely due to a better solvability and sub-sequential availability of the drug and is therefore of preference in a clinical setting. Of note in this respect, 10% ethanol could be administered to the normal murine brainstem without additional clinical or histological abnormalities. Murine studies like these will enable faster and more effective translation to the clinic and our results encourage exploring the possibility of a clinical trial studying CED of carmustine in DIPG.
Acknowledgements
We are thankful to Fred Buijs (Department of Nuclear Medicine & PET Research) for technical support with CED, to Professor Pieter Wesseling, M.D., (Department of Pathology VUMC) for histological assessment of murine brain tissue after CED, Fatma El-Khouly and René Vos (Department of Clinical Pharmacology, VUMC) for the measurements of carmustine concentration and to Thomas Würdinger, PhD., (Neuro-oncology Research Group, VUMC) for critical revision of the manuscript.

Funding
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Supplementary figure 1 | Percentage of weight loss from day 0 to day 21 of mice (n = 14) treated with CED with 10% ethanol (red line) or vehicle (blue line). No statistical difference between the groups could be detected (p = 0.41).

Supplementary figure 2 | Measurement of carmustine in vitro by HPLC–UV before and after CED procedure in both 10% ethanol (red) and 5% DW (purple) vehicle. The vehicle used caused a significant difference in concentration (p < 0.05), no difference could be noted between measurements before or after CED.

Supplemental table 1 | Characteristics of convection-enhanced delivery

<table>
<thead>
<tr>
<th></th>
<th>Clinical CED</th>
<th>Pre-clinical CED</th>
</tr>
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<tbody>
<tr>
<td>Infusion volume</td>
<td>3-9 ml</td>
<td>15 ul</td>
</tr>
<tr>
<td>Infusion velocity</td>
<td>1-5 ul/min</td>
<td>0.5 ul/min</td>
</tr>
<tr>
<td>Infusion time</td>
<td>6-100 hrs</td>
<td>30 minutes</td>
</tr>
<tr>
<td>Size pons/tumor</td>
<td>15-35 ml</td>
<td>+/- 50 ul</td>
</tr>
<tr>
<td>Weight patient</td>
<td>8-60 kg</td>
<td>20-30 gram</td>
</tr>
<tr>
<td>Concentration drug</td>
<td>No difference</td>
<td>No difference</td>
</tr>
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</table>
**Supplemental table 2 | Clinical observations after CED with 10% ethanol or vehicle**

<table>
<thead>
<tr>
<th></th>
<th>Moving</th>
<th>Walking</th>
<th>Grooming</th>
<th>Eating</th>
<th>Active behavior</th>
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<tbody>
<tr>
<td>Ethanol 10%</td>
<td>15-60 min</td>
<td>30-180 min</td>
<td>45-180 min</td>
<td>90-180 min</td>
<td>3-12 hours</td>
</tr>
<tr>
<td>Vehicle</td>
<td>25-60 min</td>
<td>45-180 min</td>
<td>60-180 min</td>
<td>90-180 min</td>
<td>3-12 hours</td>
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</table>
CED of carmustine to the murine brainstem