

A Blood-Brain Barrier Disruption Model Eliminating the Hemodynamic Effect of Ketamine

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ABSTRACT: Objective: We propose a simple modification to an established blood-brain barrier disruption (BBBD) animal model that allows us to use ketamine/xylazine as the anaesthetic agent, therefore decreasing the complexity and the cost of the model, while maintaining similar efficiency. **Methods:** Sixty-two Long Evans rats were anaesthetized by intraperitoneal injection of ketamine/xylazine. Osmotic BBBD was performed by administering 25% mannitol into the internal carotid artery in a retrograde fashion from the external carotid. The infusion rate of mannitol, as well as the duration was adjusted in a stepwise fashion to identify optimal parameters for BBBD and minimize complications. As a supplementary step to previously reported models, a vascular clip was applied to the common carotid artery prior to the infusion of mannitol, therefore isolating our model system from the depressant hemodynamic effects of ketamine/xylazine. Evans blue dye was used to control for BBBD intensity. **Results:** Using this model at an initial infusion rate of 0.15 ml/sec, a significant incidence of brain hemorrhage (75%) and a death rate of 62.5% were observed. Decreasing the infusion rate in a stepwise fashion, 0.08 ml/sec was found to produce optimal BBBD, as demonstrated by Evans blue staining. At this rate, 6/7 animals depicted grade II staining, whereas one animal depicted grade IV. **Conclusion:** The application of a clip to the common carotid artery prior to mannitol infusion allowed us to isolate the cerebral circulation from the depressant hemodynamic effects of ketamine/xylazine. This supplementary step produced consistent and efficient BBBD in our animal model.

RÉSUMÉ: Modèle de perturbation de la barrière hémato-encéphalique éliminant l'effet hémodynamique de la kétamine. Objectif: Nous proposons une modification simple à un modèle animal établi de perturbation de la barrière hémato-encéphalique (PBHE) qui nous permet d'utiliser la kétamine/xylazine comme anesthésique, ce qui diminue la complexité et le coût du modèle tout en maintenant une efficacité similaire. **Méthodes:** Soixante-deux rats Long Evans ont été anesthésiés par injection intrapéritonéale de kétamine/xylazine. La PBHE a été effectuée en administrant du mannitol 25% dans la carotide interne par injection rétrograde, à partir de la carotide externe. La vitesse et la durée d'infusion du mannitol étaient ajustées par paliers afin d'identifier les paramètres optimaux et de minimiser les complications de la PBHE. Une étape supplémentaire a été ajoutée au modèle, soit l'application d'un clip vasculaire au niveau de la carotide primitive avant l'infusion du mannitol, isolant ainsi le modèle des effets de dépression hémodynamique de la kétamine/xylazine. L'intensité de la PBHE a été évaluée par coloration au bleu d'Evans. **Résultats:** Avec un taux initial de perfusion de 0,15 ml/sec, nous avons observé un taux important d'hémorragies cérébrales (75%) et de décès (62,5%). En diminuant progressivement la vitesse d'infusion, nous avons constaté à la coloration au bleu d'Evans que le taux de 0,08 ml/sec produisait la PBHE optimale. À ce taux, 6 animaux sur 7 présentaient une coloration de grade II et 1 animal présentait une coloration de grade IV. **Conclusion:** La mise en place d'un clip au niveau de la carotide primitive avant l'infusion de mannitol nous a permis d'isoler la circulation cérébrale des effets de dépression hémodynamique de la kétamine/xylazine. Cette étape supplémentaire a induit une PBHE reproductible et efficace chez ce modèle animal.

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Chemotherapy has had little impact in the treatment of malignant gliomas of astrocytic lineage thus far.¹ The shortcomings of chemotherapy have generally been attributed to two factors, namely intrinsic glial cell resistance to chemotherapy agents, and the physiological impediment

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provided by the blood-brain barrier.² Recently, several papers have reported encouraging responses to certain chemotherapy agents in the treatment of malignant astrocytic tumors, using unconventional routes of administration.³⁻⁶ These alternate administration strategies share the goal of increasing delivery by circumventing the blood-brain barrier. Given the infiltrating nature of these neoplasms, a global delivery strategy is needed to provide a widespread penetration of therapeutic agents to the tumor, brain around tumor and infiltrated brain distant to tumor.⁷ Osmotic blood-brain barrier disruption (BBBD) stands out as a prime method to achieve this goal.⁸ Several models of blood-tumor barrier and blood-brain barrier manipulation have been developed in the animal and have been translated into clinical applications.⁸⁻¹³

Rapoport¹⁴ and Neuwelt¹⁵ were pioneers in the conception and development of the osmotic BBBD technique. This strategy was shown to be effective in many animal models prior to its use in clinical trials.^{2,3,10} The principal factors that govern the quality of osmotic BBBD are the osmolarity of the solution and the duration of exposure of the vascular endothelial cells to the hyperosmolar solution.^{2,16} Different factors can impact on the quality of the procedure, and the hemodynamic effect produced by anaesthetic agents is a fundamental variable in the equation leading to an effective and reproducible opening of the blood-brain barrier. Interestingly, the choice of the agent can have a beneficial or detrimental effect on the degree of BBBD.¹⁷ As an illustration, it was shown that the use of propofol instead of isoflurane provides a more consistent and a more intense opening of the blood-brain barrier.¹⁸

The effectiveness, reproducibility and validity of the rat BBBD model are well-documented in the literature.^{6,8,11,12,16} The use of propofol as the anaesthetic agent of choice has increased the effectiveness and the consistency of the procedure in animals. However, as reported in an earlier study, propofol can also increase the neurotoxicity of certain chemotherapy agents when used in conjunction with the established osmotic BBBD animal model.¹⁹ Moreover, the cost of propofol and the necessity of a microinfusion pump and continuous venous access for its perfusion render this model cumbersome and costly. With the ultimate goal of simplifying the model, we decided to use ketamine/xylazine because of its ease of administration as well as its cost effectiveness. Another anticipated advantage of this anaesthetic combination was the presumed absence of added toxicity as experienced by Fortin et al¹⁹ with the use of propofol. However, a previous study reported adverse effects of ketamine/xylazine on hemodynamic parameters, which contributed to an inconsistent and ineffective BBBD.¹⁷ The model described in this study was designed to eliminate the negative effect of ketamine on cerebral blood flow during the mannitol infusion in order to maintain an adequate filling of the capillary bed in the disrupted hemisphere. In an effort to accomplish this task, we designed a simple modification to the existing BBBD model that allows the elimination of the hemodynamic disturbance caused by the anaesthetic agent by isolating the perfused hemisphere from systemic circulation during the mannitol infusion. This modification simplifies the BBBD animal model while maintaining highly effective and reproducible barrier opening.

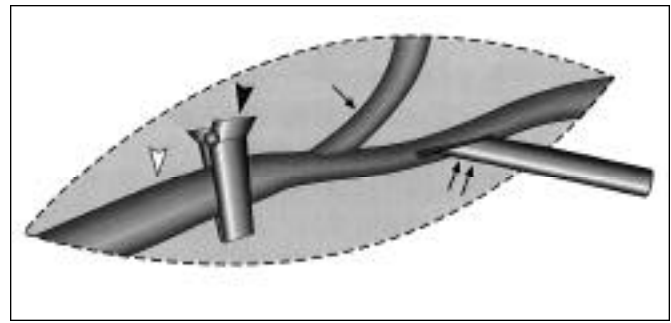


Figure: Surgical dissection of the right carotid artery complex in the rat, demonstrating the application of a temporary vascular clip (black arrow head) to the right common carotid artery (white arrow head). A PE-50 intramedic catheter (double arrow) cannulates the external carotid artery allowing infusion of mannitol in a retrograde fashion into the internal carotid artery (single arrow) to produce osmotic blood-brain barrier disruption.

MATERIALS AND METHODS

Sixty-two adult female Long Evans rats weighing 200 to 250 grams were anaesthetized with an intraperitoneal injection of ketamine (75 mg/kg) / xylazine (10 mg/kg), intubated using a 14 gauge, 2.25-inch insyte catheter and placed on an animal respirator (Harvard Apparatus Inc., Dover, Mass.) for a ventilatory support of 55 breaths/min.

All procedures were performed on a heating pad to avoid per-procedural hypothermia. A rectal probe was used to control for the body temperature. Prior to the initiation of this study, approval was obtained from the institutional animal experiment review board. As previously described,² the femoral vein was exposed and a slow IV push of Evans blue (2 ml/kg of a 2% solution in 0.9% NaCl) injected. The Evans blue was allowed to distribute within the circulating volume prior to the BBBD procedure. Using clean technique, the right carotid complex was surgically exposed and the external carotid artery was catheterized in a retrograde fashion using a PE-50 intramedic tubing so that the tip of the catheter was lying just above the bifurcation. Prior to the infusion of mannitol, a temporary vascular clip was applied to the common carotid artery approximately 1 cm proximal to its bifurcation (Figure). This simple step allowed us to isolate the perfused hemisphere from the hemodynamic effects induced by the anaesthetic agent upon the cardiovascular system. A hyperosmolar solution of 25% mannitol was then administered intra-arterially via the catheter in the external carotid artery. A constant flow syringe pump (kd Scientific model 100, USA) was used to regulate the selected infusion rate (varying from 0.15 ml/sec to 0.06 ml/sec). Once the desired dose of mannitol had been administered, the clip and the catheter were removed, and the external carotid artery was ligated. The incisions were sutured and the subjects were allowed to recover. Twenty-four hours later, the animals were sacrificed in a CO₂ chamber, and their brain extracted and examined to determine the distribution and intensity of staining

Table 1: Grading scale for descriptive quantification of blood-brain barrier disruption provided by Evan's blue staining. Scale applied on coronal slices of brain samples.

BBBD grade	Description
Grade 0	No blue staining of the cerebral parenchyma
Grade I	Slight blue tint to the cerebral parenchyma in the territory supplied by the parent artery infused with the mannitol
Grade II	Clearly demarcated blue staining of the cerebral parenchyma in the territory supplied by the parent artery infused with the mannitol
Grade III	Blue staining of the cerebral parenchyma which tends to surpass the territory supplied by the parent artery infused with the mannitol via the polygon of Willis
Grade IV	Extreme blue staining of the cerebral parenchyma which surpasses the vascular territory infused with the mannitol

produced by the Evans blue solution as a result of BBBB. To do so, the brain samples were cut in coronal slices using a brain matrix, and intensity of staining was noted and graded on the slice presenting the highest staining distribution. The grading system used is based on the qualitative description of Evans blue staining of the brain as related to the vascular territory infused by the mannitol, and has been described in detail (Table 1).^{2,19} In this study, we have arbitrarily defined grades II or III Evans blue staining of the cerebral parenchyma as adequate BBBB, grades 0 or I staining as insufficient and grade IV as excessive BBBB.

Table 2: Characteristics of the groups and sub-groups of animals treated by Osmotic Blood-Brain Barrier Disruption and a summary of the results obtained.

Mannitol rate	Duration of infusion	Status		Hemorrhage*		Degree BBBB					Total N=
		Dead	Alive	Yes	No	0	I	II	III	IV	
0.15 ml/sec	30 seconds	5	3	6	2	2	2	1	1	2	8
	25 seconds	4	2	4	2	2	0	0	4	0	6
	23 seconds	4	1	3	2	1	1	2	1	0	5
	20 seconds	0	2	1	1	2	0	0	0	0	2
0.12 ml/sec	30 seconds	4	3	4	1	0	1	4	0	0	7*
0.10 ml/sec	30 seconds	3	3	4	2	0	1	3	2	0	6
0.08 ml/sec	30 seconds	2	5	2	5	0	0	6	0	1	7
0.06 ml/sec	30 seconds	1	5	2	4	1	4	1	0	0	6
Total		23/47	24/47	26/45*	19/45*	8	9	17	8	3	47*

* Two animals were excluded from the analysis of hemorrhagic complications and degree of BBBB due to unavailable autopsy

RESULTS

A total of 62 adult female Long Evans rats initially underwent osmotic modification of the blood-brain barrier. Of these, 15 animals suffered from pre-mannitol infusion per-procedural morbidity and were not included in the analysis since blood-brain barrier modification could not be accomplished. Technical difficulties during intubation (two traumatic intubations, two barotraumas to the lungs, one disconnection of the O₂ source, two O₂ tubing leaks and two kinking of the tubing) resulted in the death of nine animals. Six other subjects did not receive the infusion of mannitol for various technical reasons: inability to cannulate the external carotid artery (n=2), catheter displacement during infusion (n=1), and pulmonary edema during the bolus injection of Evans blue thus requiring abortion of the procedure (n=3). Blood-brain barrier disruption of intra-arterial mannitol was therefore performed on 47 subjects.

The 47 animals treated were divided into sub-groups according to the infusion rate of mannitol. For technical reasons, we were unable to perform an autopsy on two of the animals. These two subjects were included for survival analysis, but were removed from the analysis of hemorrhagic complications and the quantification of BBBB grading. The sub-groups are described in Table 2.

Group 1: Infusion rate of 15 ml/sec (n=21)

A total of 21 animals were administered mannitol at a rate of 0.15 ml/sec. This rate was initially felt to be optimal for BBBB in this animal population based on the literature. The previously described models used a standard infusion duration of 30 seconds and so these parameters (infusion rate= 0.15ml/sec. and duration 30 sec) were used in a first group of eight animals. A significant death rate of 62.5 % (five of eight) was observed and variable degrees of BBBB were obtained. Of the five deaths, all but one had massive brain hemorrhage on necropsy. Two of the

three surviving animals also demonstrated brain hemorrhage. These two subjects were clinically asymptomatic.

In light of these results, we decided to alter the infusion duration in a stepwise fashion from 30 sec to the minimally accepted threshold of 20 sec (Table 2). In doing so, we anticipated a decrease in morbidity by lowering the incidence of hemorrhagic complications while improving the efficiency of BBBD. Thus a total of four sub-groups were formed at the constant mannitol infusion rate of 0.15 ml/sec, and altered infusion duration of 30 sec (n=8), 25 sec (n=6), 23 sec (n=5) and 20 sec (n=2). The results obtained in these sub-groups are summarized in Table 2. This reduction in infusion duration did not have the accounted benefit on reducing brain hemorrhage, nor did it improve the degree of BBBD. Overall, in this group of 21 animals, 13 died from the procedure (61.9%), and 14 presented a brain hemorrhage at autopsy (66.7%).

Given these results, we proceeded to alter the infusion rate of mannitol. Four additional groups were designed, keeping the infusion time constant at 30 seconds, and lowering the mannitol infusion rate in a stepwise fashion. The results are summarized in Table 2.

Group 2: Infusion rate of 0.12 ml/sec, (n=7)

In this group, the survival rate was 42.9% (three of seven). An autopsy was obtained in only two of the four animals that died following the procedure. The necropsy revealed massive hemorrhage in both cases. Autopsy of the three surviving animals revealed a brain hemorrhage in two subjects, one of which was asymptomatic. The other animal had a left hemiparesis. Consistent BBBD was obtained in all five cases where study of the brain was obtained; four animals demonstrated a grade II disruption and one, a grade I.

Group 3: Infusion rate of 0.10 ml/sec, (n=6)

The survival rate for this group was 50% (three of six). The autopsies revealed brain hemorrhage in 83.3% (five of six) of the cases; of which, three were asymptomatic. Again the degree of BBBD was fairly consistent: two grade III, three grade II and one grade I Evans blue staining were obtained.

Group 4: Infusion rate of 0.08 ml/sec, (n=7)

The survival rate was 71.4% (five of seven) in this group. At necropsy none of the surviving animals had a hemorrhagic complication and all five had grade II staining of BBBD. The autopsy of the two animals that died revealed significant brain hemorrhage in both cases. The degree of Evans blue staining obtained was a grade II in one animal and a grade IV disruption in the other.

Group 5: Infusion rate of 0.06 ml/sec, (n=6)

The survival rate for this final group was 83.3% (five of six). Hemorrhagic complications were observed in 33.3% (two of six). The Evans blue staining obtained in these subjects were less encouraging; we observed one animal with a grade II BBBD, four animals had grade I disruption, and one subject failed to show cerebral staining (grade 0).

DISCUSSION

Animal models are an indispensable tool for the development and testing of new drug therapies. A model for osmotic BBBD

has been developed in the rat, and is well-established in the literature.^{2,8} Blood-brain barrier disruption requires general anaesthesia for a number of reasons; the procedure generates a significant level of pain, causes a transient rise in intracranial pressure requiring cerebral protection, and induces hemodynamic instability. The choice of anaesthetic agent has proven important in the quality of BBBD, as well as the potential for toxicity related to the treatment drugs.¹⁷⁻¹⁹ Ketamine/xylazine, as well as other anaesthetic agents used in conjunction to the traditional BBBD model, was found to be inconsistent, producing only 40-70% of good to excellent BBBD. It is presumed that negative impact on the cardiac index, as well as on cardiac rate and systemic arterial pressure reduces the effectiveness of the procedure by altering cerebral blood flow, thus decreasing the effective rate of mannitol delivery during the intra-arterial infusion.¹⁸ Relative inconsistencies in BBBD obtained when using certain anaesthetic agents prompted efforts to modify the technique. Propofol has been shown to be the most efficient agent, producing greater than 95% of good to excellent BBBD in animals.¹⁸ With increase in both the consistency and the intensity of the BBBD, propofol has been found to produce neuro-toxicity with certain chemotherapy agents not previously reported as toxic with other anaesthetics.¹⁹ Hence the choice of a specific anaesthetic agent may be accompanied by undesirable toxicity eliminating any potential advantages.

The principal factors governing the quality of BBBD are the osmolarity of the solution and the duration of exposure of the vascular endothelial cells to the hyperosmolar solution.^{2,16} The application of a temporary vascular clip to the common carotid artery (CCA) during mannitol infusion allowed us to isolate the cerebral hemisphere from hemodynamic effects of the anaesthetic agents, rendering the mannitol infusion independent of the cardiac output. This simplifies the hemodynamic system by decreasing the number of variables involved. The rate and duration of infusion then become the sole relevant modifiable parameters. In the present study using ketamine/xylazine as the anaesthetic agent, and applying a temporary vascular clip to the CCA, we have been able to obtain consistent BBBD. The optimal rate of mannitol infusion was found to be 0.08 cc/sec. Using this rate of mannitol infusion, a balance was reached between the primary endpoint (BBBD) and complications. However, the application of a vascular clip and its related consequences on the infusion dynamics of the system are not without adverse effects. The initial infusion rate of mannitol (0.15 ml/sec) resulted in a significant incidence of brain hemorrhage (66.7%) with a corresponding death rate of 61.9%. Although no control group was used in this study, preliminary results obtained in our lab using the standard technique, without clip application, and ketamine as an anaesthetic, displayed poor BBBD results (two grade 2, one grade 1 and five grade 0) at an infusion rate of 0.12 ml/sec, but no hemorrhagic complications (n=8 animals). We tried decreasing the duration of mannitol infusion in order to eliminate this complication. The modification effectively reduced the incidence of cerebral hemorrhage from 75% to 50%, but it also decreased the efficiency of BBBD. We assume that the shorter exposure of the vascular endothelial cells to mannitol account for this decrease in intensity of BBBD.¹⁶ Although the number of animals in each subgroup is small, the decline in BBBD seen in these figures lead

us to believe that reducing the duration of mannitol infusion below 30 seconds would not be beneficial. Most investigators also agree that 30 seconds is the critical period necessary for BBBB.²

The next logical step was to vary the rate of infusion, while keeping the duration constant at 30 seconds. The effective standardized rate of infusion in the rat model has been established by previous investigators at 0.12 ml/sec for isoflurane, and 0.09 ml/sec for propofol.¹⁸ Given the inadequate results reported by other investigators with the use of ketamine/xylazine at an infusion rate of 0.12 ml/sec for this procedure in the traditional animal model,¹⁷ and based on our preliminary data, we opted to initiate the study at an infusion rate of 0.15 ml/sec. At this rate, we found an unacceptable high rate of mortality due to brain hemorrhage. The decrease in mortality observed by altering the infusion parameters, associated with the decrease in hemorrhages found at autopsy, supports this hypothesis. We presume that the hemorrhage is a consequence of the incapacity of the vessels to accommodate the high flow delivered. This results in an increase in intraluminal pressure leading to hydrodynamic stress which surpasses the compliance of the capillaries causing them to rupture. In the absence of the vascular clip, the increased pressure generated by the infusion of mannitol is released as back flow in the CCA. This back flow, although protecting the cerebral circulation from the increased hydrodynamic pressure, may produce systemic complications, such as pulmonary edema or cardiac failure,¹⁹ due to reflux of mannitol into the heart and pulmonary circulation. By reducing the infusion rate, we reduced the intraluminal pressure generated and the hydrodynamic stress, and thus were able to dramatically reduce the incidence of brain hemorrhage (33%). The mortality rate nevertheless remains relatively high regardless of the significant reduction from 62.5% to 16.7%. However, BBBB is an invasive technique, causing hemodynamic variations, and hence could be expected to cause mortality in an animal model where cardiovascular parameters were not carefully monitored in this pilot study. Evidently there may be a learning-curve phenomenon which partially explains this decrease in mortality. We expect to reduce the death rate below 10% with increased experience and additional modifications to the infusion parameters. Moreover, we have recently acquired equipment to monitor precisely and continuously the hemodynamic parameters.

The efficiency of BBBB produced by other model systems approaches 95% in normal rats using isoflurane or propofol as the anaesthetic agent.¹⁸ Infusion of mannitol at a rate of 0.15 ml/sec produced only 43% (9/21) good to excellent BBBB, and one third (7/21) of the animals failed to show Evans blue staining. Six of these grade 0 BBBB occurred in animals having died a short time after the procedure. We believe that their precocious deaths allowed insufficient time for the Evans blue to equilibrate across the interrupted blood-brain barrier. Indirect evidence supporting this statement comes from the other groups where, as the death rate decreased, so did the number of grade 0 disruptions observed. As the infusion rate is decreased towards 0.08 ml/sec, we see an increase in the proportion of adequate BBBB, producing 6/7 grade II (85.7%) and one grade IV. No insufficient (grade 0 or grade I) BBBB occurred at this infusion rate. However, as the rate is reduced further (0.06 ml/sec), the

efficiency of BBBB seems to fall off producing only 16% adequate disruptions (1/6). These results suggest that an infusion rate of 0.08 ml of mannitol/sec is the inferior limit for adequate osmotic BBBB in this animal model while maintaining morbidity at an acceptable level. We attribute the ability to reduce our infusion rate so low and yet still maintain efficient BBBB to the application of a temporary vascular clip on the CCA during administration of hyper-osmolar intra-arterial mannitol. This model is cheaper, less cumbersome and just as reliable as the models previously reported.

We describe a simple modification to a previously established animal model for osmotic BBBB. The application of a temporary vascular clip to the CCA during the procedure allowed us to isolate the cerebral circulation from the potential hemodynamic effects of ketamine/xylazine as anaesthetic agents. This supplementary step produced consistent and efficient disruption of the blood-brain barrier. Clipping the CCA also prevented back flow of mannitol into the pulmonary circulation and by eliminating the hemodynamic influences of the anaesthesia on the cardiac index, blood pressure and pulse rate, allowed us to reduce the infusion rate of mannitol. The reduced rate of infusion requires a lower volume of mannitol and therefore minimizes the complications related to volume overload. This new model is as effective, easy to perform and less costly than previously reported models.

GRANT INFORMATION

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