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A new method of quantitatively assessing the opening of the blood–brain barrier in murine animal models

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ABSTRACT

The blood–brain barrier (BBB) restricts the delivery of drugs into the brain. Different strategies have been developed to circumvent this obstacle. One such approach, the osmotic BBB disruption (BBBD), has been under pre-clinical study since the 70's. Typically, qualitative *ex vivo* assessment of the extent of BBBD has been performed using Evan's blue staining technique. In this study, we describe a simple quantitative technique based on albumin indirect immunohistochemistry to measure the extent of BBB breach. Thirty Fischer rats were assigned to one of 6 groups: a control group, and BBBD groups with escalation in IA mannitol infusion rate: 0.06, 0.08, 0.10, 0.12 and 0.15 cc/s. Fifteen minutes after the BBBD procedure, the animals were sacrificed, brain harvested and sections stained for albumin. Using an image analysis software, isolated albumin staining pixels were expressed as a fraction of the treated hemisphere. This ratio was used as a percentage value in the intensity of the BBB permeabilization. All sections studied harbored staining, averaging 0.37% for the controls (group 1), 5.69% for group 2 (0.06 cc/s), 10.44% for group 3 (0.08 cc/s), 6.99% for group 4 (0.1 cc/s), 18.50% for group 5 (0.12 cc/s) and reaching 61.70% for group 6 (0.15 cc/s). Important variations were observed between animals. A threshold effect was observed, and animals in group 6 presented a significant increase in BBB permeabilization compared to the other groups. We hereby detail a simple technique that can be applied to quantitatively measure the extent of the BBB breach notwithstanding the pathological process.

1. Introduction

The BBB is a complex physiological entity located at the level of the cerebral endothelial cell presenting a surface area of approximatively 20 m^2 in the human. The BBB derives its restrictive function in delivery from multiple anatomical and physiological characteristics, such as the presence of tight junctions, the expression of different efflux pumps, a luminal negative charge of the endothelial cells, the presence of basal lamina and of the astrocytic podophilic projections. Altogether, the BBB limits the passage of water soluble molecules presenting a molecular weight greater than 180 Da (Kroll et al., 1996; Pardridge, 2005). In fact, it is estimated that 98% of all the therapeutic molecules cannot reach the brain parenchyma in pharmacologically-relevant concentrations (Neuwelt et al., 2008). This 'neurovascular unit' thus impacts the treatment of different pathologies by greatly limiting the entry of therapeutic molecules and restricting the arsenal at our disposal. As a consequence, a significant number of molecules with the activity *in vitro*, such as antineoplastic agents against glioma cell lines, have limited efficacy because of limited brain bioavailability *in vivo*. Therefore, the concept of transitorily breaching the permeability or the function of the BBB to increase delivery of therapeutics is highly relevant (Boyle et al., 2004; Bradford et al., 1997). Different approaches have been developed to circumvent the BBB, such as intra-arterial infusion of hyperosmolar solutions, infusion of bradykinin receptor agonists and convection enhanced delivery (Kraemer et al., 2002). The intra-arterial infusion of hyperosmolar solutions, or blood–brain barrier disruption (BBBD), has been studied and characterized, both in clinical and pre-clinical studies (Blanchette et al., 2009; Fortin, 2003, 2004; Fortin et al., 2005; Fortin and Neuwelt, 2003; Kraemer et al., 2002; Kroll and Neuwelt, 1998; Neuwelt, 1989, 1980; Neuwelt et al., 1980, 1986; Pardridge, 2005). Our clinical team commonly uses this approach in the treatment of primary CNS lymphoma, malignant gliomas and brain metastasis (Fortin et al., 2005, 2007).

The BBBD procedure involves the intra-arterial infusion of a hyperosmolar solution (mannitol 25%) to produce a transient

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increase in the permeability of the BBB. This is typically followed by the intra-arterial infusion of the therapeutic agents (*e.g.* chemotherapy). The effectiveness of the procedure can be influenced by different factors, including hemodynamic variables, type of anesthesia and rate of hyperosmolar solution infusion (Fortin et al., 2004; Remsen et al., 1999). It is thus paramount to monitor the degree of the barrier permeabilization obtained after a procedure, as it can be highly variable from patient to patient, and even with repeated procedures in the same subject.

In the clinic, a CT scan with an IV radiographic contrast agent infusion is performed after a BBBD procedure to evaluate the degree of the BBB permeabilization in a semi-quantitative fashion, based on an analog scale. The validity of this monitoring approach has been established (Neuwelt et al., 1980; Roman-Goldstein et al., 1994). Using this technique, some authors have reported an association between the degree of the BBBD as a surrogate of drug delivery, and survival of patients bearing primary central nervous system lymphomas treated with the BBBD procedure (Kraemer et al., 2001).

In pre-clinical studies however, the most widely used approach to study the extent of the BBBD process is an *ex vivo* technique requiring the intravenous infusion of a 2% solution of Evans blue prior to the BBBD (Fortin, 2003, 2004; Kroll and Neuwelt, 1998). This *in vivo* marker binds tightly but reversibly to albumin, which does not normally cross the BBB (Neuwelt, 1989). Thus, the increase in the BBB permeability allows diffusion of the albumin-Evans blue complex into the brain parenchyma. Once the brain is harversted after the BBBD procedure, the intensity distribution of the blue coloration in the treated hemisphere can than be qualitatively assessed, and a visual score is attributed (Fortin, 2003). The qualitative and subjective aspect of this approach decreases the reliability of this evaluation method (Fortin, 2003, 2004).

Recently, we developed a new technique to monitor the dynamic process of the BBBD by DCE-MRI in pre-clinical research (Blanchette et al., 2009) that possesses the invaluable advantage of allowing *in vivo* assessment. However, not all research groups have access to an animal MRI scanner in their facilities, and thus *ex vivo* qualitative or semi-quantitative techniques to evaluate the efficacy of the BBBD procedure remain pertinent and useful.

To improve the knowledge of the BBBD process, its evaluation has to be revisited. A quantitative measurement of the degree of BBBD would allow a better analysis in the intensity of delivery against efficacy in any given experiment regarding CNS treatments. Accordingly, in the design of new glioma treatment strategies, it is essential to distinguish between the different factors at play, such as the delivery impediment and the efficacy of the therapeutic molecule. Only by isolating and analyzing theses two variables individually will we be able to optimize the design of new treatment approaches. It is common knowledge that albumin does not cross the normal BBB, and that it is easily labeled by indirect immunohistochemistry. The aim of this study was thus to develop a simple, accurate and quantitative technique to measure the intensity of delivery produced by the BBBD procedure.

2. Materials and methods

The experimental protocol was approved by the institutional ethical committee and conformed to regulations of the Canadian Council on animal care.

2.1. Study groups

Thirty adult male Fischer rats weighing 250 g were obtained and kept under controlled conditions in our facilities. The animals were randomly assigned to one of 6 groups (Table 1). Group 1 acted as the control group, and was exposed to an intra-arterial saline infusion as a sham BBBD procedure. Groups 2–6 were treated using the BBBD technique (Fortin et al., 2000, 2004) at different rates of intra-arterial mannitol infusion, in a stepwise increment fashion, from 0.06 to 0.15 cc/s (0.06, 0.08, 0.10, 0.12 and 0.15). Typically, a rate of 0.12 cc/s is adequate to produce a BBBD in these animals (Fortin et al., 2004).

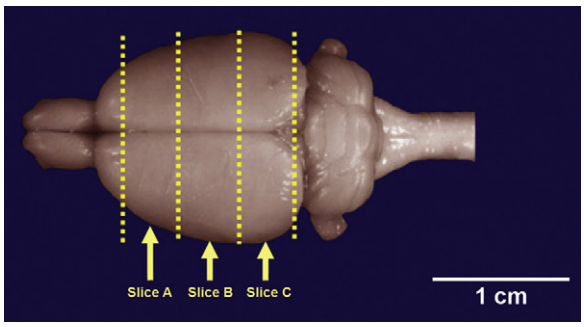

Fig. 1. Schematic illustration showing the 3 areas as defined and cut using the brain matrix. The section B was used for the study.

2.2. BBBD procedures

Procedures were performed under general anesthesia using an intra-peritoneal injection of ketamine (87 mg/kg) and xylazine (13 mg/kg). Endotracheal intubation was performed under direct visualization of the trachea with a 18G insyte catheter. Using an aseptic technique, the right carotid complex was surgically exposed and the right external carotid artery was catheterized in a retrograde fashion with a PE-50 polyethylene tubing, so that the tip of the catheter was lying just above the bifurcation. Prior to mannitol infusion, a clip was applied on the common carotid artery to limit backflow of the administered solution (Fortin et al., 2004). Mannitol (25%) was then infused using a micro-infusion pump, at the rate prescribed by the study group to which the animals were assigned, for a constant infusion time of 30 s (Table 1).

2.3. Evans blue staining, brain harvest and albumin indirect immunohistochemistry

For comparison purpose, 1 animal from each group was also infused with a 2% solution of intravenous Evans blue (2 ml/kg), prior to the BBBD procedure. Fifteen minutes after the procedure, while still under general anesthesia, the animals were sacrificed by an intracardiac formaldehyde perfusion, and brains were harvested and preserved for 48 h in formaldehyde solution. The brain specimens were cut in 3 coronal sections using a brain matrix and section B was embedded in paraffin (Fig. 1). The paraffin-embedded blocks were cut in 5 μm coronal slices with a microtome. Albumin indirect immunohistochemistry was performed, using a goat IgG antibody fraction against rat albumin (1:200, MP Biomedicals, OH, USA) incubated overnight at 4 °C in a humid chamber. Sections were incubated 1 h with HRP-conjugated mouse anti-goat antibody (1:100, GE Healthcare, Buckinghamshire, UK) at room temperature in a humid chamber. Both antibodies were diluted in TBS containing 10% non-fat powdered milk. As a negative control, a set of sections was not exposed to the primary antibody. Albumin detection was revealed by diaminobenzidene (DAB) (Roche, Qc, Canada). An hematoxylin counterstaining was performed; slides were then dried, mounted and analyzed.

Table 1
Animal groups.

Groups	1	2	3	4	5	6
Infused solution	Saline	Mannitol	Mannitol	Mannitol	Mannitol	Mannitol
Infusion rate (cc/s)	0.12	0.06	0.08	0.10	0.12	0.15
n	4	4	4	4	4	4[a]

[a] An animal was excluded due to the mortality during the procedure.

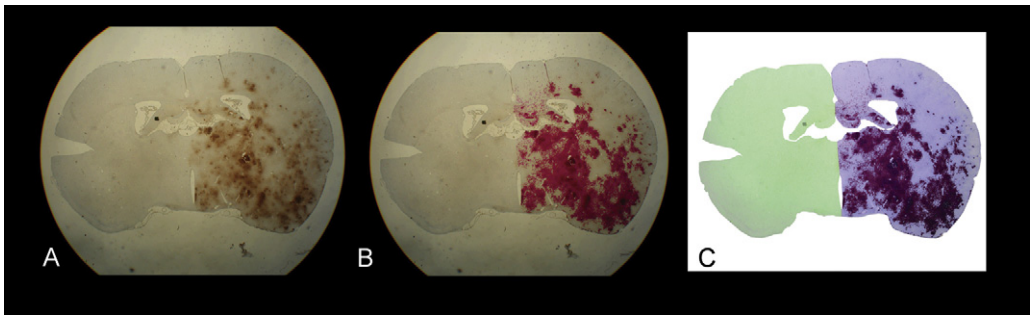

Fig. 2. Description of the steps involved in the calculation of the intensity of delivery ratio used in this study. (A) The albumin indirect immunohistochemistry source image, displaying discolored areas in the treated hemisphere. (B) The image has been analyzed to identify pixels above a fixed threshold corresponding to the immunohistochemistry staining. These pixels are retained as the red overlay. C) After having defined the pixel area of each hemisphere (green overlay is left hemisphere, whereas blue overlay is right hemisphere), the red overlay has been added to the image for final analysis. Results are expressed as number of stained pixels (red overlay) as a fraction (%) of the treated (right) hemisphere (blue overlay).

2.4. Image analysis

All sections were digitized and processed using the software Sigma Scan Pro, version 5 (Hallogram Publishing, Aurora, USA). Briefly, the outlining of the brain parenchyma was manually drawn for each hemisphere, taking care to exclude the ventricles and subarachnoid spaces. The area defined in this manner was converted into the number of pixels. Albumin indirect immunohistochemistry produces a brownish discoloration that can be isolated from the hemisphere using an intensity threshold set to retain the pixels above a certain value (Fig. 2A and B). The number of stained pixel in the treated hemisphere was calculated (Fig. 2C), and reported as the percentage of stained pixels over the treated hemisphere. This ratio value was used as an indicator of the intensity of delivery. Statistical analyses between the six groups were performed using a one-way ANOVA ($p < 0.0001$) followed by a Dunnett's Multiple Comparison test ($\alpha < 0.05$).

3. Results

Mean staining values obtained for each animal are presented in Fig. 3. As can be appreciated from this data, all groups depicted significantly different staining values. As expected, the saline infused animals (group 1) presented insignificant albumin staining, with an average of staining ratio of 0.37%. Animals from groups 2–6 presented increased staining when compared to the control group (Fig. 3). However, this difference was statistically significant only when mannitol was administered at a rate of 0.15 cc/s (group 6, $p < 0.0001$). As can be appreciated from the standard deviation shown in Fig. 3, a significant inter-individual variation was observed within each group. Two animals from group 5 displayed very low staining, thus significantly lowering the average intensity measured in that group. As the BBBD process has typically been considered an all or none phenomenon, this hints at the possibility that the osmotic threshold was not reached at 0.12 cc/s in these two animals.

Fig. 4 presents a composite figure of a representative animal from each group depicting Evans blue staining of the brain surface, and a corresponding coronal slice. In this figure, significant staining can be appreciated for animals of groups 5 and 6, but not so for the animals included in groups 2–4. Using the albumin staining described herein, we found permeabilization percentage mean values of 5.69% (group 2), 10.44% (group 3) and 6.99% (group 4) respectively.

4. Discussion

Osmotic BBBD is one of the strategies designed to bypass the blood–brain barrier, and improve delivery of therapeutic molecules to the central nervous system. The aspect of delivery is underemphasized in the literature, and has not always received the proper attention it deserves (Pardridge, 1997, 2005). Even now, delivery across the BBB is overlooked as an important cause of failure in the treatment of many CNS diseases (Fortin et al., 2005). Admittedly, researchers working in the field of delivery have not yet been able to establish a firm relationship between the extent of delivery and a potential clinical benefit for the patient, whether it is in neuro-oncology, or in other CNS pathologies. Kraemer and al. are the only group that has so far addressed the relationship between intensity of delivery and the outcome, in a population of patients bearing primary CNS lymphomas (Kraemer et al., 2001).

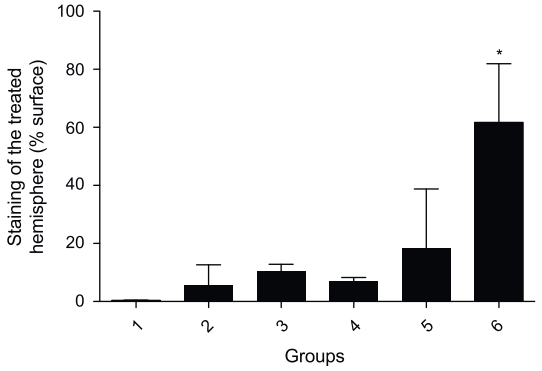

Fig. 3. Graphic expressing the staining percentage of the treated hemisphere obtained for each group (mean ± SD). The mean of each group was significantly different ($p < 0.0001$). Only group 6 was significantly different from the control group (*, $p < 0.05$).

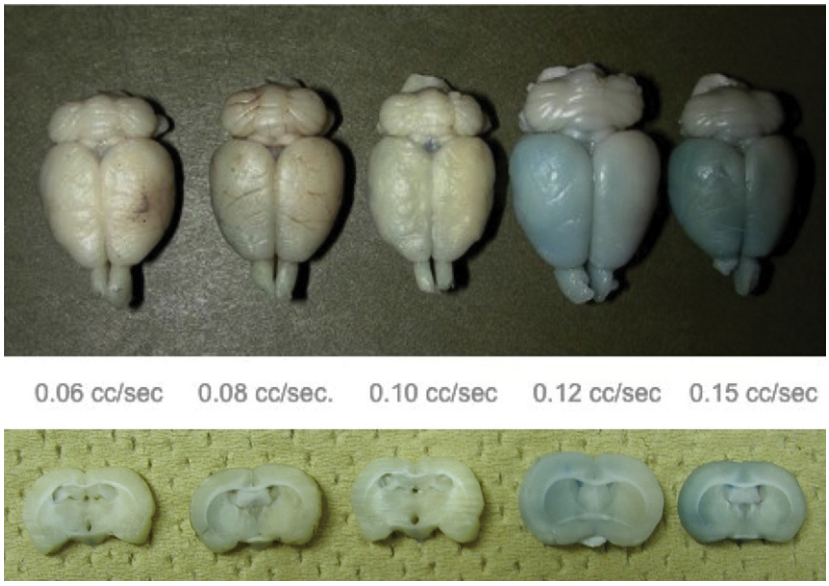

Fig. 4. Whole brains and corresponding coronal slices of samples extracted from one representative animal of each group exposed to different rates of mannitol infusion and an Evans blue IV administration. A sequential increment in discoloration is observed and a maximal coloration is reached at 0.15 cc/s.

In that paper, a relationship was established between the extent of delivery, as a function of the intensity and the number of BBBD procedures, and the survival of patients. Recently, our group has shown a prolongation in the median survival of patients with primary and secondary brain tumors treated by way of BBBD procedures to increase chemotherapy delivery (Fortin et al., 2005). Unfortunately, these clinical studies were not randomized in design, and thus bear inherent weaknesses prohibiting a firm conclusion as to the relevance of BBBD in impacting favorably the outcome of patients. As pre-clinical studies have convincingly demonstrated the potency of the procedure to increase delivery of chemotherapy and of various therapeutic molecules across the BBB, the demonstration that this increase in delivery impacts tumor response and patient survival now needs to be established. Consequently, pre-clinical studies testing new drug candidates must correlate the drug delivery with the treatment efficiency using surrogates as tumor size and survival. The usual histological techniques to monitor the opening of the BBB are either qualitative or semi-quantitative (Evans blue). The Evans blue technique involves the infusion of an IV Evans blue solution prior to the BBB manipulation. The marker binds albumin, which has a 60 kDa molecular weight, and thus typically does not cross the BBB (Fortin, 2003). Once the procedure is performed, the animal is euthanized and the brain harvested, an analog scale-based evaluation of the intensity and distribution of the bluish coloration is used to evaluate the degree of the BBB permeabilization. Needless to say that this approach involves a certain degree of subjectivity. Radioactivity can also be used to grossly monitor the extent of delivery (Bhattacharjee et al., 2001). This strategy implies an exposure to radioactivity and remains semi-quantitative, limiting the conclusion when studying the contribution of the extent of delivery against other variables such as survival.

The present report details a simple and reproducible technique that allows collection of objective data mirroring the extent of delivery, based on albumin indirect immunohistochemistry. This allows the production of a conservative estimate on the extent of delivery, as albumin is a large protein. This estimate is expressed as a percentage of the treated hemisphere on a given coronal slice, and can also be used as a composite score reflecting global delivery, by simply summating the scores obtained on multiple contiguous slices, enabling spatial distribution studies. As the brain samples are cut in a standardized fashion using a brain matrix, the number of slices is always consistent, thus ensuring that the composite score is reproducible.

This concept has already been exploited in the past by Vorbrodt et al. (1994) in the study the dynamics of BBBD. These authors used quantitative immunogold labelling against albumin in scanning electron microscopy. The numerical value was based on the square micrometer density of gold particles, and was used to detail the dynamics in the BBB permeabilization across different compartments. However, as the image analysis was performed on electron micrographs, the method described by Vorbrodt et al. did not allow a global estimation of delivery such as the analysis we performed.

The evaluation technique we describe herein could be useful in the assessment of new CNS delivery techniques, as well as in the study of pathologies that induce increases in BBB permeability such as stroke or inflammatory conditions. However, in these pathologies the BBB permeability does not extend to the overall studied hemisphere; thereby the staining ratio has to be corrected for focal edema using the formula developed by Swanson and his colleagues (Swanson et al., 1990).

The major drawback of this approach is the fact that it cannot be accomplished *in vivo*, and still requires the sacrifice of the animal. As we have developed and reported a technique allowing the real-time monitoring of the BBBD permeability and permiting the evaluation in the intensity of delivery by DCE-MRI (Blanchette et al., 2009), we would evidently suggest the use of this *ex vivo* static approach as an alternative monitoring technique whenever an *in vivo* approach cannot be deployed.

As delivery strategies to the CNS are being developed and increasingly used, the ability to measure the improved delivery of different molecules should be reproducible, easy to perform and allow quantification to become a validated method of measurement. Investigators also need to demonstrate that this increase in delivery impacts other surrogates, such as response and survival, before the concept of increased delivery across the BBB can be considered a standard of treatment. The simple technique described in this paper allows an evaluation of the extent of BBB breach notwithstanding the cause, whether it be iatrogenic (BBBD *via* different means) or pathological (inflammation, tumor or ischemia).

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Conflict of interest

The authors declare no conflict of interests.

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