Assessment of Blood–Brain Barrier Disruption in Stroke

Andrea Kassner, PhD; Zamir Merali, MD

The disease burden of acute ischemic stroke (AIS) is rising in the United States and Canada. It is estimated that in the United States 795,000 people have a stroke each year, which amounts to $34 billion in medical expenses and lost productivity. Recent trials have shown the potential benefit of procedural thrombectomy in the management of AIS. However, despite substantial research expenditures over the past 2 decades, there have been no new pharmacological agents for the initial treatment of AIS since the approval of recombinant tissue-type plasminogen activator in 1996. Recently, the role of the blood–brain barrier (BBB) in the pathogenesis of AIS has emerged as a focus for new therapeutic strategies. After the onset of AIS, the BBB is rapidly disrupted and this disruption persists for days through the acute and early subacute phases of AIS. The disruption of the BBB can be quantified as an increase in the permeability of the BBB (Figure 1). In the setting of AIS, disruption of the BBB is thought to be a precursor to serious clinical consequences such as hemorrhagic transformation (HT), which refers to the development of a hemorrhage within the ischemic brain tissue. Current research is attempting to elucidate the mechanisms of BBB disruption after AIS and develop therapeutic strategies to mitigate the clinical consequences of BBB disruption.

Given these developments, a greater number of preclinical and clinical studies are incorporating assessment of BBB disruption into their study designs. Although assessment of BBB disruption may once have been technically difficult, a plethora of methods now exist to assess BBB disruption with relative ease in animal models and humans. When taken together, these methods cover a wide range of temporal and spatial resolutions and permit simultaneous assessment of cell morphology, protein expression and localization, cell electrophysiology, and gross neurological function. An awareness of the specifications and limitations of the methods of assessing BBB disruption is necessary to design effective studies and evaluate the literature.

In the present review, we summarize the available methods of assessing BBB permeability in animal models and humans. We strive to highlight the relative strengths and weaknesses of these methods and provide suggestions for study design in stroke research. Finally, we discuss recent translational developments in assessing BBB disruption for the treatment of AIS.

Ex Vivo Assessment of BBB Disruption
The pioneering experiments of Max Lewandowsky first defined the BBB as a dynamic barrier separating the intravascular and extravascular compartments. This laid the groundwork for current methods of assessing BBB disruption in AIS animal models and human trials. All methods of assessing BBB disruption are common in their use of tracers, which can be either endogenous if they are naturally found in the blood or exogenous if introduced into the circulation by the experimenter. Table I in the online-only Data Supplement summarizes methods of assessing BBB disruption.

Measurement of Blood Proteins
A straightforward and common method of assessing BBB disruption is the measurement of extravasated blood proteins in the brain parenchyma, using immunofluorescence or immunohistochemistry (Figure 2A). Albumin, fibrinogen, IgM, and IgG can be assessed with this method. In a rat model, lower background staining can be achieved by using antirabbit antibodies directly conjugated to horseradish peroxidase (HRP). An advantage of this method is that no exogenous tracer is introduced into the circulation, eliminating potential confounding factors. In addition, paraffin-embedded tissue samples allow simultaneous assessment of morphological changes and cell death. A disadvantage of assessing endogenous tracers is that subtle BBB disruptions may be missed because of the large size of most endogenous tracers. In addition, measuring the extravasation of endogenous tracers does not assess BBB disruption at a single time point, but rather provides a summation of blood protein extravasation since the time of ischemic injury.

Measurement of Exogenous Dyes
Numerous exogenous tracers are available with varied molecular weights (MWs) and chemical properties. The extravasation
kinetics of many exogenous tracers are known, making them versatile tools to quantify BBB disruption across a range of injury severities.

The blue azo dye, Evans blue, has a MW of 1.0 kDa. A notable property of Evans blue is its propensity to bind serum albumin. The dye can, therefore, be used to measure the extravasation of albumin (66.5 kDa). In the simplest method, BBB disruption can be assessed qualitatively by simple visualization of blue discoloration within sectioned brain tissue. Alternatively, the dye can be extracted from brain tissue by incubating in formaldehyde and quantifying dye concentration with ultraviolet spectrophotometry. Jaffer et al recently described ex vivo assessment of Evans blue extravasation with an optical imaging assay. With an optical imaging assay, the amount of Evans blue extravasation can be quantified in a brain slice, a region of interest, or the whole brain. Optical imaging can achieve high sensitivity when compared with ultraviolet spectrophotometry. A drawback of measuring Evans blue extravasation by any method is the risk of overestimating BBB permeability to albumin because of the dye’s reversible binding kinetics with serum albumin. In addition, measurement of Evans blue extravasation cannot be used to determine extravasation kinetics.

Free dyes (having low binding affinity to serum proteins) are available in a wide range of MWs. The dextran amines (5.0–120.0 kDa) are particularly versatile because they can be coupled to a wide variety of light compounds such as biotins or fluorophores. Careful dye formulation permits injection of dextran dyes with multiple MWs within a single experiment and separate quantification ex vivo. When the extravasation kinetics and tissue volumes of distributions of small exogenous tracers (eg, free fluorophores, gadolinium, and dextran dyes) were compared with those same tracers coupled to compounds with high MW, the apparent severity of BBB disruption was found to be dependent on the size of the tracer. BBB disruption was also seen to be inhomogeneous across brain regions and evolve with time. These findings demonstrated that BBB disruption after AIS did not possess an all-or-nothing characteristic as others had suggested, and indicated that using a single-sized exogenous tracer might not be sufficient to fully characterize BBB disruption after AIS.

HRP (MW 44.0 kDa) is a free dye well suited for simultaneous assessment of BBB disruption and cellular morphology. Injection of HRP and subsequent tissue preparation and incubation with diaminobenzidine cause a pigmentaion that can be visualized macroscopically and microscopically. Figure 3B illustrates HRP extravasation through microvessels under electron microscopy, which permits extremely high-resolution assessment of cell–cell interactions and allows visualization of the intracellular localization of the tracer. A disadvantage of HRP is that it cannot be used to determine extravasation kinetics.

Small free dyes such as HRP and fluorophores can be linked to serum proteins such as albumin or immunoglobulin and pharmacological agents to assess their transfer through the BBB. Coupling of HRP to pharmacological agents permits a detailed analysis of the pharmacological agent’s ability to cross the BBB. Many dyes can be measured in the brain parenchyma semiquantitatively with microscopy or through optical density measurement. It is also possible to extract free dyes from brain tissue and measure their concentrations fluorometrically or photometrically. However, even with fluorometric or photometric assessment, extravasation kinetics cannot be ascertained.

Quantitative autoradiography allows for the derivation of the extravasation kinetic parameters of BBB disruption while maintaining reasonably high spatial resolution (50–200 μm). Quantitative autoradiography studies have used numerous radiolabelled compounds as tracers such as the amino acid [14C] α-aminoisobutyric acid and [4-18F] fluorooantipyrine. Custom radiolabelled compounds can be prepared to assess the transfer of specific compounds across the BBB. Figure 2C depicts autoradiograms from rats that were subjected to focal ischemia/reperfusion and given a bolus of 14C α-aminoisobutyric acid. When analyzed against the experimental plasma concentration time–curve of 14C α-aminoisobutyric acid, a permeability constant was derived with high signal to noise ratio. A disadvantage of quantitative radiography is that the technique requires specialized equipment, and many protocols take multiple weeks to complete.

In Vivo Assessment of BBB Disruption

Microscopic Assessment

Multiphoton and 2-photon microscopy provide an in vivo method of assessing BBB disruption on surface microvasculature with high spacial and temporal resolution. Abulrob et al demonstrated the assessment of BBB disruption in cortical surface vessels after experimental AIS. After undergoing focal ischemia and reperfusion, rats were anesthetized and a craniotomy was made over the desired cortical location. Numerous fluorescent tracers are available with short plasma half-lives (eg, indoline derivatives, sodium fluorescein, Lucifer-Yellow). Microscopic cortical imaging and subsequent image analysis
allows measurement of BBB disruption and simultaneous measurement of cerebral blood flow, leukocyte behavior, and cell death. Assessment of BBB disruption with fluorescent microscopy typically achieves high temporal resolutions of 30 frames/second. A modification of this method used 99technetium-gluceptate with a γ-camera instead of a fluorescent tracer.29

An advantage of assessing BBB disruption by fluorescence microscopy is that pharmaceutical agents can be given systemically or topically, and their effect on the BBB can be measured in real time. This method can be combined with measurement of evoked neuronal responses permitting correlation of BBB disruption with neuronal activity. Disadvantages of assessing BBB disruption by fluorescent microscopy are that animal subjects must be under anesthesia, which may exert a time-dependent effect on BBB disruption and blood pressure.30 In addition, visualization is typically limited to superficial vasculature because of poor tissue penetration and light scattering. However, increased penetration can be achieved with the use of near-infrared light.26

A novel extension of assessing BBB disruption by fluorescence microscopy is the use of an implantable closed cranial window (Figure 2D).28 After surgical implantation of the closed cranial window, rats are given 1 to 2 weeks to recover and can then undergo experimental focal ischemia and reperfusion. Implantation of a closed cranial window permits long-term (≤4 weeks) evaluation of microcirculatory parameters...
such as BBB disruption, cerebral blood flow, and leukocyte behavior. As rats do not need to be kept under anesthesia during observation of the microcirculation, anesthetic effects on BBB disruption are eliminated. However, this method is also limited to visualizing surface vasculature. Assessment of BBB disruption by fluorescence imaging may have translational potential although this potential requires further study.

Assessment of BBB Disruption in Humans

Only few methods can be safely incorporated into the typical clinical management of AIS. The following methods, suitable for the use in clinical studies, have also been used in preclinical trials, making them attractive choices for translational preclinical study designs.

Assessment of BBB Disruption With Magnetic Resonance and Computed Tomography Imaging

Magnetic resonance (MR) imaging and computed tomographic (CT) imaging are the most widely used clinical imaging modalities/tools to evaluate BBB disruption by detecting the extravasation of intravenously administered low MW contrast media. In its simplest form, BBB disruption can be assessed qualitatively, by determining the presence or absence of contrast enhancement on structural images of the brain. When dynamic contrast-enhanced (DCE) MRI or CT is combined with a suitable pharmacokinetic model, one may quantify and spatially map BBB disruption throughout the brain.

DCE-MRI typically involves intravenous bolus injection of a gadolinium contrast agent followed by the repeated acquisition of T1-weighted images of the brain (Figure 3A–3C). Then, assuming a linear relationship between image MR signal intensity as a function of time (SI(t)) and contrast agent concentration as a function of time (C(t)), one can generate a set of concentration versus time curves for each voxel or region of the brain. One also typically generates at least one curve consisting exclusively of blood plasma data (Cp(t)). Evidence of enhancement indicates that contrast material has escaped the confines of the intravascular compartment via dissection and Cp(t) data sets and estimate BBB permeability in standard units of mL/100 g per minute. These concepts can be similarly applied to DCE-CT. Whereas DCE-MRI involves intravenous injection of a paramagnetic contrast agent and voxel-wise measurement of MR signal intensity as a function of time, DCE-CT involves intravenous injection of an iodinated contrast agent and voxel-wise measurement of attenuation coefficient (Hounsfield units) as a function of time (Figure 4A and 4B).

In many cases, the pharmacokinetic models that are now applied to DCE-MRI and DCE-CT data were originally developed for nuclear medicine tracers. Most of these are compartmental models, which define the tissue space as a volume with both intravascular and extravascular compartments. These models define parameters that describe the exchange of contrast media between the blood plasma and the tissue extracellular compartment. Those parameters of particular interest are the transfer (rate) constant/efflux parameter (Ktrans) and the extravascular distribution volume (Ve), which corresponds to the size of the extravascular extracellular space. These parameters describe physiological measures, making them attractive for monitoring changes in the cerebral vasculature during treatment and determining the surrogate end points for treatment in longitudinal comparisons and multicenter trials.

No matter what pharmacokinetic model is selected, it is assumed that the tracer extravasation fraction is low relative to blood flow, to avoid a flow-limited scenario and to permit interpretation of modeled parameters in terms of vascular permeability. This introduces a considerable limitation to the use of clinically approved low MW contrast agents. In most tissues, the first-pass extraction fraction of these agents is high (30%–70%) violating the assumption and limiting interpretability to apparent permeability. Within the brain, however, the BBB restricts extravasation of even these tracers allowing kinetic modeling in terms of BBB permeability to characterize BBB disruption in AIS.

Alternatively, a limited number of studies have considered model-free approaches to deriving permeability parameters.
in patients with AIS.\textsuperscript{39,40} Model-free approaches simplify data analysis and may therefore be easier to incorporate into a clinical setting. For example, measurements derived from the relative-recirculation method were shown to correlate significantly with $K_{\text{trans}}$ values derived from a Patlak model applied to DCE-MRI.\textsuperscript{39}

In the clinical setting, the availability and speed of CT make it the modality of choice when making treatment decisions for AIS.\textsuperscript{41-48} BBB disruption can potentially be assessed by incorporating a DCE-CT protocol into the initial CT imaging of a patient with AIS. To avoid repeated radiation exposure associated with DCE-CT, clinical studies may be able to combine an initial DCE-CT scan with DCE-MRI for reassessment, as the 2 modalities were shown to produce comparable $K_{\text{trans}}$ values.\textsuperscript{45}

**Translational Potential: BBB Assessment in the Clinic**

The prediction of HT is an active area of research that may refine clinical decision making in AIS and make thrombolytic therapy available to patients who are currently excluded. Several studies have shown that the extent of BBB disruption as assessed with DCE-MRI correlates with the risk of developing HT.\textsuperscript{4,41-48} A multicenter study with 296 patients incorporated DCE-MRI into a multivariable model to predict the risk of HT with high sensitivity and specificity.\textsuperscript{5} The therapeutic window of recombinant tissue-type plasminogen activator is currently constrained to 4.5 hours because of the risks associated with delivering recombinant tissue-type plasminogen activator at a later time point. Through the assessment of BBB disruption, it may be possible to stratify patients by their individual risk of developing HT and perhaps administer recombinant tissue-type plasminogen activator safely to selected patients beyond 4.5 hours. Several unanswered questions remain. There is currently no consensus in AIS around which pharmacokinetic model is the preferred one for the derivation of $K_{\text{trans}}$, or if a model-free approach may produce similar results more simply. Clinical studies to date have had modest sample sizes and a larger multicenter study or meta-analysis may be necessary to gather larger sample sizes and determine the efficacy of individualized risk assessment.

**Conclusions**

We have outlined available methods of assessing BBB disruption in preclinical and clinical studies of AIS. Given the growing number of studies examining the BBB in the context of AIS, an understanding of these methods is now important for the researcher to possess. In preclinical study models, numerous diverse methods allow for assessment of BBB disruption combined with assessment of cell morphology, protein expression and localization, cell electrophysiology, or gross neurological function. In clinical trials of AIS, MR and CT permeability imaging are the main methods by which BBB disruption can be assessed safely. One promising clinical application of BBB assessment is the use of permeability imaging as a prognostic tool to predict an individual patient’s risk of HT. Examination of the BBB after AIS has proven to be a fruitful research direction with the potential to improve the clinical management of AIS.

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**Disclosures**

None.

**References**

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Supplemental Table I – A comparison of the methods for assessing blood-brain-barrier disruption in pre-clinical models and humans with key citations highlighted. (N = no, Y = yes).

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