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Far-red tracer analysis of traumatic cerebrovascular permeability

George P. Liao, MD,* Scott D. Olson, PhD, Daniel J. Kota, PhD, Robert A. Hetz, MD, Philippa Smith, BA, BS, Supinder Bedi, PhD, and Charles S. Cox Jr., MD

Department of Pediatric Surgery, University of Texas Health Science Center at Houston, Houston, Texas

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ABSTRACT

Background: Blood brain barrier (BBB) compromise is a key pathophysiological component of secondary traumatic brain injury characterized by edema and neuroinflammation in a previously immune-privileged environment. Current assays for BBB permeability are limited by working size, harsh extraction processes, suboptimal detection via absorbance, and wide excitation fluorescence spectra. In this study, we evaluate the feasibility of Alexa Fluor 680, a far-red dye bioconjugated to dextran, as an alternative assay to improve resolution and sensitivity.
Methods: Alexa Fluor was introduced intravenously on the day of sacrifice to three groups: sham, controlled cortical impact (CCI), and CCI treated with a cell based therapy known to reduce BBB permeability. The brains were sectioned coronally and imaged using an infrared laser scanner to generate intensity plot profiles as well as signal threshold images to distinguish regions with varying degrees of permeability.
Results: Linear plot profile analysis demonstrated greater signal intensity from CCI than treated rats at corresponding injury depths. Threshold analysis identified rims of signal at low + narrow threshold ranges. The integrated signals from a treatment group known to preserve the BBB were significantly less than the groups with CCI injury alone. There was no significant difference at high + wide signal intensity threshold ranges.
Conclusions: Alexa Fluor 680 infrared photodetection and image analysis can aid in detecting differential degrees of BBB permeability after traumatic brain injury and maybe particularly useful in demonstrating BBB preservation of at-risk regions in response to therapeutic agents.

1. Introduction

Severe traumatic brain injury (TBI) defined as a Glasgow Coma Scale of 3–8 continues to carry high morbidity in the pediatric and adult population with limited diagnostic and treatment modalities. Blood Brain barrier (BBB) permeability and cerebral edema play crucial roles in the pathophysiological progression of secondary injuries after TBI, and are clinically manifest as increased intracranial pressure and reduced cerebral perfusion pressure. BBB compromise leads to vasogenic edema, exposing the previously immunoinflammatory-privileged cerebral tissue to an influx of inflammatory factors and cells [1]. Cytotoxic edema coexists and is the result of the cellular insult and the loss of the ability to maintain intracellular volume [2].

* *Corresponding author*. Department of Pediatric Surgery, University of Texas Health Science Center, 6431 Fannin, MSB 5.230, Houston, TX 77030. Tel.: +1 713 500 7300; fax: +1 713 500 7296.
E-mail address: George.P.Liao@uth.tmc.edu (G.P. Liao).
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The injury penumbra has been previously described by our laboratory and collaborators as tissue adjacent to the area of injury, and characterized by the presence of neuroinflammatory cells and proinflammatory cytokines [3]. The condition of the BBB in this at-risk region of brain is likely in transition between healthy and dysfunctional cerebral microvasculature and can be evaluated in the preclinical setting via excess tissue water (cerebral edema) and dye extravasation (increased microvascular permeability). Clinically, BBB compromise can be estimated via microdialysis catheters or external ventricular drains as ratios between brain and serum total protein or albumin levels [4]. However, direct preclinical and clinical visualization of penumbral BBB permeability *in situ* has been limited to imaging modalities such as diffusion tensor magnetic resonance imaging (MRI). Active research exists in identifying and targeting therapy to rescue these at-risk regions.

In preclinical studies, the simplest method of indirectly assessing the degree of BBB dysfunction is brain water content, which offers global differences and is subject to variations in animal sacrifice technique and post mortem handling of brain tissue [5]. Evans Blue dye extravasation, a popular assay used to study the BBB response after TBI has affinity to the 66 kDa albumin. Thus under normal circumstances, Evans Blue dye remains in circulation because of the 0.4 kDa pore size of the BBB. After TBI, BBB compromise causes Evans Blue extravasation into the interstitial tissue and can be quantified after extraction [6–8]. We, along with our collaborators have previously used tissue water, Evans Blue, and immunohistochemistry to demonstrate that cell-based therapies can modulate the neuroinflammatory response after TBI resulting in penumbral BBB preservation [9,10]. Of note, Pati and colleagues demonstrated in mouse models of TBI that mesenchymal stem cells exert perivascular protective effects to the penumbral BBB via increased junctional protein (vascular endothelial - cadherin and occludin) expression [11,12]. Walker *et al.* showed increased localization and organization of occludins to the microvasculature [13].

Despite wide use in TBI research, the utility of Evans Blue is limited due to several factors. The first is the relatively large molecular size of albumin compared with the BBB pore size. Second, the detection of extracted Evans Blue uses absorbance, a suboptimal detection process complicated by a wide excitation fluorescence spectrum. Third, the harsh extraction process destroys brain tissue and precludes further histologic analysis on the same tissue sample.

Alexa Fluor (Life Technologies, Carlsbad, CA) is a fluorescent marker that has been conjugated to a variety of molecules and has been used in investigations for drug transit across the BBB [14]. Highly sensitive, high-resolution infrared laser scanners can detect subtle changes in signal intensities of Alexa Fluor between adjacent anatomical structures in brain tissue samples. We have previously demonstrated that Alexa Fluor reduces the nonspecific signal between sham and injury to 7% ($P < 0.001$), an eight-fold reduction (Fig. 1). The high sham signal for Evans Blue is likely due to the tissue handling and the extraction process where extraparenchymal and intravascular Evans Blue dye cannot be easily excluded from intraparenchymal dye when placed under an absorbance microplate reader. The goal of our study was to evaluate Alexa Fluor 680, a far-red dye bioconjugated to dextran (10 kDa) as an alternative, to Evans Blue to expand research capabilities with a multimodal detection assay that increases the sensitivity to BBB perturbations, allowing the identification and quantification of at-risk penumbral areas of microvascular injury, and preserving tissue for further assays.

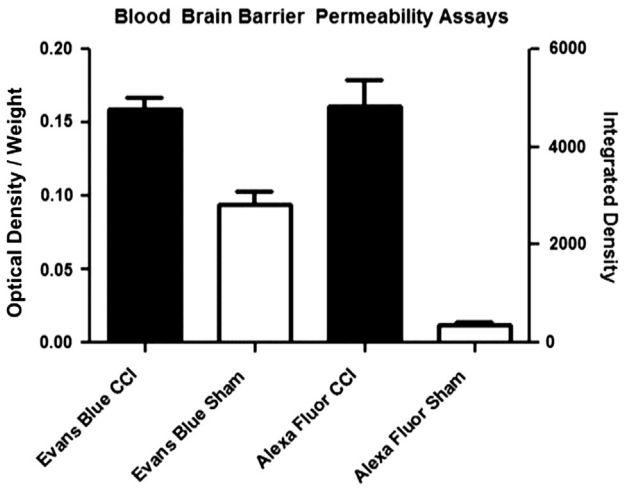

Fig. 1 – Nonspecific signals are reduced with Alexa Fluor compared with Evans Blue dye as evidenced by the high signal from sham animals receiving Evans Blue. Evans Blue CCI ($n = 11$), Evans Blue sham ($n = 6$), Alexa Fluor CCI ($n = 9$), Alexa Fluor sham ($n = 10$). The *y*-axis Optical Density/Weight refers to optical density (absorbance) per rat weight. The *x*-axis integrated density refers to the cumulated signal detected by the far-red scanner across the brain slices.

2. Materials and methods

All protocols involving the use of animals were in compliance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the University of Texas Institutional Animal Care and Use Committee (protocol HSC-AWC-11-120).

2.1. *Surgical study design*

The three groups in this study were sham ($n = 10$), controlled cortical impact (CCI) ($n = 10$), and treatment ($n = 10$). All groups received Alexa Fluor intravenously at the time of euthanasia. Each group underwent our acute TBI model, undergoing their respective injury at time zero followed by euthanasia at 72 h. In this study, the treatment group received human adult bone marrow-derived stromal cells that we previously showed by Evans Blue and tissue water assays to reduce post TBI BBB permeability [9,10]. The treatment group received the cell-based product at 24 and 48 h after injury via tail vein injection.