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Quantitative and Qualitative Assessment of Glymphatic Flux Using Evans Blue Albumin

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Abstract

Background: The glymphatic system is a proposed pathway for clearance of proteins and macromolecules from brain, and disrupted glymphatic flux is implicated in neurological disease. We capitalized on colorimetric, fluorescent, and protein-binding properties of Evans blue to evaluate glymphatic flux.

New method: Twenty-five μ L of 1% Evans blue-labeled albumin (EBA) in artificial cerebrospinal fluid (aCSF) was injected into the intracisternal space of anesthetized postnatal day 17 rats. Serum was collected at various time points after injection ($n=37$) and EBA was measured spectrophotometrically. In separate rats ($n=3$), a cranial window was placed over the parietal cortex and EBA transit was evaluated using *in vivo* multiphoton microscopy. Separate rats ($n=6$) were processed for immunohistochemistry to examine localization of EBA. In some rats, intracranial pressure (ICP) was increased via intracisternal injection of aCSF.

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Conflict of Interest

The other authors have no conflicts of interest to declare.

Results: EBA was detected in serum as early as 30 min, was maximal at 4 h, and was undetectable at 72 h after intracisternal injection. Using intra-vital microscopy and immunohistochemistry EBA could be tracked from CSF to perivascular locations. Consistent with removal via glymphatic flux, increasing ICP to 40 mmHg accelerated transit of EBA from CSF to blood.

Comparison with existing methods: Transit of EBA from CSF to serum could be quantified spectrophotometrically without radioactive labeling. Glymphatic flux could also be qualitatively evaluated using EBA fluorescence.

Conclusion: We present a novel technique for simultaneous quantitative and qualitative evaluation of glymphatic flux in rats.

Keywords

Blood-brain barrier; Bulk flow; Glymphatics; Lymphatics

1. Introduction

The glymphatic system is a recently re-characterized perivascular pathway for clearance of interstitial fluid (ISF), solutes, and waste from the brain. In the 1980's, Cserr and colleagues observed that radiolabeled albumin injected into brain parenchyma or cerebrospinal fluid (CSF) concentrated in the lymphatic vessels of the neck and cervical lymph nodes (Bradbury et al., 1981; Whalen et al., 1999), and also that a number of large molecular weight tracers including Evans blue albumin (EBA) accumulate in perivascular spaces after intracerebral injection (Ichimura et al., 1991; Szentistványi et al., 1984). More recent work by Iliff et al. used fluorescently labeled protein tracers injected into CSF to show flux of labeled proteins into brain interstitial fluid through a perivascular pathway lined by the foot processes of astrocytes, exiting the brain along perivenous channels into extracranial lymphatics (Louveau et al., 2015). This system is referred to as “glymphatics,” given the glial boundary and its function analogous to the lymphatic system in other organs (Iliff et al., 2014). It is proposed that macromolecule solutes move through the perivascular space via a bulk flow mechanism, possibly driven by cerebral arterial pulsation (Iliff et al., 2013) and facilitated diffusion of water via aquaporin-4 channels. Alternatively, diffusion-mediated CSF/ISF exchange may clear macromolecule substrates along venous perivascular spaces independent of aquaporins (Abbott et al., 2018).

Removal of protein and other macromolecules is important in maintaining brain homeostasis and is implicated in various physiologic and pathophysiologic states. This process is accelerated during sleep, and slowed by aquaporin-4 gene deletion, acetazolamide administration, and germane to the experiments in this report, low intracranial pressure (ICP) induced by cisterna magna puncture (Plog et al., 2015). Impaired waste clearance has been postulated to contribute to progression of neurodegenerative disorders (Peng et al., 2016). Suppressed glymphatic flux in aquaporin-4 knockout mice results in reduced clearance of amyloid beta from brain parenchyma (Iliff et al., 2014) and increased neurofibrillary formation and neurodegeneration in an animal model of traumatic brain injury (TBI) (Plog et al., 2015). These findings suggest that the glymphatic system plays an

important role in development of Alzheimer's disease and chronic traumatic encephalopathy. An animal model of type-2 diabetes showed reduced interstitial fluid clearance in the brain, suggesting impaired glymphatic flux as a mechanism for diabetes-related cognitive impairment (Jiang et al., 2016). The glymphatic system may also be relevant in diagnosis of brain injury. It is suggested that biomarkers of TBI are carried to the bloodstream via the glymphatic system, and alterations in this flow may affect the detectability and/or specific blood or CSF levels of clinically relevant markers of disease (Plog et al., 2015).

Given the important role in clearing macromolecule waste products, and its implication in numerous acute and chronic brain insults, we sought to establish a simple method for visualizing perivascular flow and quantifying macromolecule transit from CSF to blood. Accordingly, we capitalized on the colorimetric, fluorescent, and protein-binding properties of EBA to evaluate *in vivo* these processes consistent with glymphatic flux.

2. Materials and Methods

2.1. Intracisternal injection of Evans blue albumin

Evans blue (EB) is an azo dye with both fluorescent (excitation 620 nm, emission 680 nm) and colorimetric properties. It binds with very high affinity to albumin (Saria and Lundberg, 1983), and has been used classically as an intravenous injection to evaluate blood-brain barrier (BBB) permeability (Whalen et al., 1999). 100 mg of EB (Sigma-Aldrich, St. Louis, MO) was added to 100 mg of rat albumin (Sigma-Aldrich, St. Louis, MO) diluted in 10 mL artificial cerebrospinal fluid (aCSF) to make a 1% solution of EBA.

All experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and all surgical procedures were performed using aseptic technique. Post-natal day 17 Sprague-Dawley rats were anesthetized with 2% isoflurane/N₂O/balance O₂, tracheally intubated and mechanically ventilated to control arterial blood concentrations of oxygen and carbon dioxide. Post-natal day 17 rats were chosen in anticipation of future studies in pediatric models of cardiac (Fink et al., 2004) and TBI (Jenkins et al., 2002; Kochanek et al., 2017). Femoral arterial and venous catheters were placed by surgical cutdown for continuous mean arterial pressure (MAP) measurement, frequent arterial blood sampling, and administration of drugs. For intracisternal (i.c.) injection of EBA and monitoring and increasing ICP, PE-10 tubing was inserted into the cisterna magna via dural puncture and placement confirmed by aspiration of CSF. The catheter was secured in place with tissue glue, dental cement, and a skin suture. A pressure transducer was attached for continuous monitoring of ICP. Next, 25 μ L of 1% EBA was injected into the cisterna magna. In a subset of rats, ICP was increased to 40 mmHg for 30 min via controlled infusion of aCSF (0.1–0.2 mL/min) using an infusion pump to reach and maintain target ICP. Heart rate, MAP, and ICP were measured continuously. Rectal temperature was measured continuously and maintained at 37 ± 1 °C with a heating pad. Blood gases were obtained to ensure rats had a PaCO₂ of 30–40 mmHg and a PaO₂ of 80–120 mmHg at baseline. If necessary, the ventilator and/or FiO₂ were adjusted and blood gases repeated. For 4, 24, and 72 hour timepoints, catheters were removed after ICP returned to baseline and anesthesia was discontinued. The endotracheal tube was removed when rats began spontaneously breathing and rats were returned to their cages after recovery. All rats appeared to tolerate the

procedure including EBA injection and increased ICP (if applicable) and there was no late mortality.

2.2. Quantification of Evans blue albumin in serum

Serial blood samples from rats were collected at specified times via the femoral artery catheter, and centrifuged for 10 minutes at $2000 \times g$ to isolate serum. After sacrifice, brains were harvested and placed in 3 mL of formamide (Sigma-Aldrich, St. Louis, MO) to allow EB to diffuse from brain tissue. EBA concentrations were measured in serum and formamide samples using spectrophotometry (620 nm) against a standard curve generated with solutions of known EBA concentration in aCSF or formamide.

2.3. Qualitative evaluation of Evans blue albumin using in vivo multiphoton microscopy

Animal preparation for *in vivo* microscopy was performed using a method adapted from a recent study of the rat cortical microvasculature (Li et al., 2017), with addition of an i.c. catheter for administration of EBA and aCSF, and monitoring of ICP (Figure 1A). Rats were tracheally intubated, anesthetized, mechanically ventilated, and had i.c. catheters placed as described above. Femoral arterial and venous catheters were placed. End-tidal CO_2 was monitored to ensure CO_2 levels of 30–40 mmHg since a blood gas machine was not readily available in the Center for Biological Imaging. To ensure immobility during in-vivo multiphoton microscopy, rats received vecuronium (5 mg/kg/h intravenously) for neuromuscular blockade. Body temperature was maintained at 37°C with a heating pad. Rats were positioned prone with the head stabilized in a stereotactic frame. The skull was exposed via midline scalp incision, and a 3-mm cranial window was made with a dental drill over the parietal cortex. A solution of 1% agarose in 0.9% saline was applied to the intact dura, and a 5-mm diameter glass coverslip was applied and secured with tissue glue and dental cement.

A Nikon A1R microscope was configured with the Nikon 1-E (Nikon Instruments, Tokyo, Japan) upright motorized system. The microscope was configured with a Chameleon Laser Vision (Coherent, Inc., CA, USA), and an APO LWD 25 water immersion objective with 1.1NA, a high-speed (30 frames/sec) resonant scanning mode and a galvano-mode scanning suitable for linescanning acquisitions. The Nano-Drive system was used to acquire fine high-speed control of z-plane selection. The fluorescence detection unit of the microscope consisted of four detectors (photo multiplying tubes). We used detector 2 for fluorescein isothiocyanate (FITC) 525/50 nm (green channel) and detector 4 for EB 650/720nm (red channel). The microscope components including the laser, stage, resonant scanning head, detectors and acquisition were controlled using NIS Elements software (Nikon). To identify blood vessels, we administered 100 μL 3% (w/v) FITC-conjugated dextran (wt. 2000 kDa, Sigma-Aldrich, St. Louis, MO, USA) IV at baseline. Additional injections were given as needed to maintain adequate delineation of pial arterioles running perpendicular to penetrating arterioles. The microscope stage was enclosed in a temperature controlled black plexiglass chamber, which prevented interference by the ambient light. We used an excitation wavelength of 850 nm and a laser power of 1%. ICP was increased to 40 mmHg by infusing aCSF via the i.c. catheter 30 minutes after EBA injection, maintained at 40

mmHg for 30 min, then allowed to return to baseline. Image stacks were obtained at baseline and up to 75 minutes after EBA injection.

2.4. Qualitative evaluation of Evans blue albumin using immunohistochemistry

Rats were tracheally intubated, anesthetized, and mechanically ventilated as above. 25 μ L of 1% EBA in aCSF was injected i.c. in rats with or without increasing ICP to 40 mmHg for 30 minutes as described above. One hour after EBA injection, rats were sacrificed, transcardially perfused with ice-cold 5% heparin in 0.9% saline, followed by 2% paraformaldehyde (PFA) in 1 $\times$ phosphate-buffered saline (PBS). Brains were harvested and post-fixed in 2% PFA for 2 hours, and cryopreserved in 30% sucrose solution in 1 $\times$ PBS for 48 hours.

Brains were cut in sagittal sections (5 μ m) and washed three times with PBS, followed by 3 $\times$ washes with solution of 0.5% bovine serum albumin (BSA) in PBS. Sections were blocked with 5% normal goat serum in BSA solution for 45 minutes. To label lymphatic vessels and astrocytes the slides were incubated for 1 hour at room temperature with rabbit anti-lymphocyte vessel endothelial hyaluronan receptor 1 (LYVE1; 1:200, ab14917, Abcam, Cambridge, UK) and chicken anti-gial fibrillar acidic protein (GFAP; 1:500, ab4674, Abcam, Cambridge, UK), respectively, in a 0.5% BSA solution. Slides were washed three times with BSA solution and incubated for 1 hour at room temperature with Alexa 488 donkey anti-chicken secondary antibody (1:500, 703–545-155, Jackson Immuno, West Grove, PA, USA) combined with donkey anti-rabbit CY3 (1:1000, 711–165-152, Jackson Immuno, West Grove, PA, USA) in BSA solution. Nuclei were stained with bisbenzamide (1 mg/100 ml water) for 30 seconds. After three rinses with PBS, sections were coverslipped using gelvatol mounting media. Large area scan images were captured with a Nikon A1 confocal microscope (NIS Elements 4.4).

2.5. Statistics

Quantitative data are presented as mean $\pm$ standard error of the mean (SEM). Changes in serum EBA levels over time were compared using ANOVA with Sidak's post hoc test. Differences in serum EBA levels and physiologic variables between groups and over time with ICP raised to 40 mmHg vs. control ICP were compared using two-way ANOVA with Sidak's post hoc test (GraphPad Prism version 7.00 for Macintosh, GraphPad Software, La Jolla, California, USA). A P -value < 0.05 was considered statistically significant.

3. Results

3.1. Transit of Evans blue albumin from cerebrospinal fluid to serum

EBA was detected in serum from rats as early as 30 minutes (0.9 ± 0.6 μ g/mL) after i.c. injection, reaching levels of 4.3 ± 0.1 and 2.0 ± 0.3 μ g/mL at 4 and 24 hours, respectively in control animals (Figure 1B; $^{\dagger}P < 0.02$ vs. 15 minutes; $n = 4$ /timepoint). Increasing ICP to 40 mmHg for 30 minutes accelerated movement of EBA from CSF to serum in rats, reaching levels of 2.5 ± 0.6 , 3.9 ± 0.9 , and 3.6 ± 0.2 μ g/mL at 15, 30, and 60 minutes, respectively (Figure 1B; NS; $n = 4$ –5/timepoint). Serum EBA was higher in rats when ICP was increased to 40 mmHg compared with control rats at 15, 30, and 60 minutes (Figure 1B; $*P < 0.05$).

Physiologic variables ICP, MAP, and heart rate measured in these rats are shown in Table 1. Other than ICP, there were no differences between groups or compared with baseline values over time. However, we did not attempt to control blood pressure as ICP was our target variable, and this experiment may have been underpowered to determine an impact of alterations in MAP in transit of EBA from CSF to serum.

3.2 Gross distribution of Evans blue after intracisternal injection

EBA was grossly visible on the surface of brains after sacrifice, most clearly apparent along the paths of major cerebral arteries and at the base of the brain (Figure 1C). This distribution was similar to previous reports where EBA was injected into perivascular spaces (Ichimura et al., 1991). Perivascular EBA was more prominent in rats where ICP was increased to 40 mmHg after i.c. injection. EB was also assessed in brains stored in formamide for 72 hours to extract the EB from the tissue. The concentration of EB in formamide measured spectrophotometrically was 12.2 ± 2.2 , 13.3 ± 3.7 , 9.2 ± 1.7 , and 4.5 ± 1.1 $\mu\text{g/mL}$ formamide at 1, 4, 24, and 72 hours, respectively ($n = 4/\text{group}$).

3.3 Perivascular distribution of Evans blue albumin identified using multiphoton in vivo microscopy

EBA was also visualized utilizing the fluorescence properties of EB via a cranial window in rats using in vivo microscopy. Three rats were first used to establish imaging parameters for EBA without increasing ICP. Image stacks were obtained from 3 rats at baseline and up to 75 min after EBA injection. Representative images collected at 30, 60, and 75 minutes after EBA injection with corresponding ICP measurements are shown in Figure 1D. EBA (red) was seen in the perivascular space surrounding pial arterioles. FITC-conjugated dextran was injected as an intravascular contrast agent (green).

3.4 Regional distribution of Evans blue albumin after intracisternal injection and impact of raised intracranial pressure defined using large area scan images

Figure 2 shows the regional distribution of EBA using large area scan images from representative rats ($n = 3/\text{group}$). In control rats 2 hours after i.c. injection, EBA (white) was detected in periventricular and perivascular spaces (Figure 2). LYVE1-positive lymphocytes (yellow) were occasionally observed in approximation to GFAP-positive astrocyte end feet (green) and EBA in perivascular spaces, consistent with lymphatic/glymphatic channels. Increased EBA extravasation into the brain emanating from periventricular and leptomeningeal spaces was observed after ICP was increased to 40 mmHg via i.c. infusion of aCSF (Figure 2). Increased perivascular EBA intensity was also observed after ICP was increased. These data along with serum EBA measurements suggest accelerated transit of EBA from CSF to blood via perivascular routes, consistent with glymphatic channels, with increased ICP.

4. Discussion

To better understand the movement of macromolecules through the brain we developed a novel and simple *in vivo* method using the well-known albumin-binding dye EB to follow protein transit from CSF to blood. The colorimetric and fluorescent properties of EB allow

for its use in spectrophotometric quantification, as well as fluorescence microscopy. We found that EBA is detectable in blood as early as 30 minutes after i.c. injection, with a peak at 4 hours. Increasing ICP to 40 mmHg for 30 minutes accelerates appearance of EBA in blood. We speculate that the hydrostatic pressure exerted on the CSF space provides a driving force for bulk flow of CSF and/or CSF/ISF exchange along a perivascular route consistent with a glymphatic pathway. This is indirectly consistent with observations made by Plog et al. where reducing ICP via craniotomy inhibited glymphatic flux (Plog et al., 2015). Increasing ICP was the primary variable for establishing validity of our methods; however, we cannot disentangle the potential impact of increased blood pressure vs. ICP in accelerating transit of EBA (Table 1). It will be valuable in the future to explore the impact of graded thresholds of ICP and MAP on EBA transit from CSF and ISF (via intraparenchymal injection) to blood.

Further evidence of the perivascular route of protein transit from CSF to interstitial fluid to blood was demonstrated using *in vivo* multiphoton microscopy and large area scan images and immunohistochemistry. In real time, EBA can be observed within perivascular spaces in living animals after i.c. injection. Large area scan images showed EBA flux from the ventricular and leptomeningeal CSF spaces into adjacent brain parenchyma. The qualitative observation of deeper brain penetration of EBA after increased ICP lends support to the mechanistic suggestion that hydrostatic pressure drives flow through the perivascular space via bulk flow and/or increased diffusion-mediated CSF/ISF exchange (Abbott et al., 2018). Our results are consistent with earlier work by Cserr et al. using radiolabeled albumin to evaluate quantitative protein flux from brain to lymph nodes, as well as EBA to qualitatively evaluate protein flux from brain parenchyma to perivascular locations (Ichimura et al., 1991; Szentistványi et al., 1984). More recent work by Iliff et al. used fluorescently labeled protein tracers to define the glymphatic system (Iliff et al., 2014), and serum brain biomarkers to indirectly assess glymphatic flux after TBI (Plog et al., 2015). Recently, CSF flow was evaluated using fluorescent tracers including EB in a mouse model of subarachnoid hemorrhage (Golanov et al., 2018). However, to our knowledge the current study is the first to directly quantify transit of high molecular weight tracers (EBA) from CSF to blood. This method represents a simple and parsimonious approach to delineating perivascular flux of macromolecules from CSF/ISF to blood, by combining many useful aspects of previously published methods, and may be useful in further examination of the glymphatic (Iliff et al., 2014) or alternative pathways (Abbott et al., 2018), including a possible role in experimental brain injury.

This method provides the added benefit of quantifying large molecule efflux from the brain. EB has been traditionally used as an intravenous injection to evaluate BBB integrity, given that the intact BBB is impermeable to EB-bound proteins including albumin. We took advantage of these properties in this method. Spectrophotometry allows for quantification of glymphatic flux from brain to serum without the need for radioactive tracers. Glymphatic flux assays could be used to lend further insight to serum biomarker assays in animal models of brain injury (Kochanek et al., 2016). Using an EBA assay to compliment serum biomarker findings in various models of brain injury and neurological disorders could contribute to improved understanding of brain injury, particularly in models which do not typically result in BBB permeability to albumin, such as global ischemia from cardiac arrest

(Tress et al., 2014). However, experimental models that severely disrupt the BBB might limit the utility of this assay in some cases. In that setting, direct transit of EBA from brain to blood and reuptake of EBA from the blood into the brain might occur across a damaged BBB via mechanisms distinct from glymphatic flux, potentially making inferences regarding the glymphatic system more challenging in such models.

5. Conclusion

We present a novel technique for simultaneous quantitative and qualitative analysis of protein flux from CSF to blood *in vivo*. EBA, a fluorescently and colorimetrically labeled protein, can be visualized entering the brain in real-time along blood vessels at the pial surface, penetrating the brain interstitium, and quantified in serum. Our findings are consistent with the glymphatic hypothesis for clearance of macromolecules from the brain. The method presented in this report may be useful for the evaluation of glymphatic and/or alternative macromolecular clearance pathways in normal animals and in animal models of injury and disease.

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Abbreviations:

(aCSF)	Artificial cerebrospinal fluid
(BBB)	Blood-brain barrier
(BSA)	Bovine serum albumin
(CSF)	Cerebrospinal fluid
(EB)	Evans blue
(EBA)	Evans blue albumin
(FITC)	Fluorescein isothiocyanate
(GFAP)	Glial fibrillary acidic protein
(ISF)	Interstitial fluid
(ICP)	Intracranial pressure
(i.c.)	Intracisternal
(LYVE1)	Lymphocyte vessel endothelial hyaluronan receptor 1

(MAP)	Mean Arterial Pressure
(PBS)	Phosphate buffered saline
(TBI)	Traumatic brain injury

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Research Highlights

- Transit of Evans blue albumin from CSF to serum measured spectrophotometrically
- Transit of Evans blue from CSF to perivascular spaces visualized in real time
- Simultaneous quantitative and qualitative analysis of glymphatic flux

Figure 1. Transit of Evans blue albumin (EBA) from cisterna magna to serum.

A. Preparation for *in vivo* microscopy including catheter placed in cisterna magna for injection of EBA, infusion of artificial CSF, and measurement of intracranial pressure (ICP), and cranial window overlying the left parietal cortex. **B.** Quantification of EBA in serum of control rats (blue, open circles) and rats where ICP was increased to 40 mmHg for 30 minutes (red, squares). *Inset:* serum sample taken from a rat 24 hours after increasing ICP to 40 mmHg for 30 minutes showing visibly blue color. Mean $\pm$ SEM, (*n*); **P* < 0.05 vs. control at same timepoint; †*P* < 0.02 vs. 15 minutes. **C.** In control rats EBA is seen grossly

along the paths of major arteries on the base of the brain 1 hour after injection. When ICP is increased to 40 mmHg for 30 min EBA extends dorsally along blood vessels. **D.** Perivascular distribution of EBA (red) observed using in multiphoton *in vivo* microscopy along pial arterioles visible with intravenous FITC-conjugated dextran (green). Representative images obtained from image stacks (n = 3 rats) with time and corresponding ICP. A 3-dimensional image stack from the 30 minute time point with the colors separated is provided below.

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Figure 2. Regional distribution of Evans blue albumin (EBA) after cisterna magna injection. Representative ($n = 3/\text{group}$) multicolor large area scan images in 5 μm sagittal brain sections labeled with EBA (white), anti-GFAP antibodies to identify astrocytes (green), anti-LYVE1 to identify lymphocytes (yellow), and DAPI to identify cell nuclei (blue). **A.** In a control rat 2 hours after injection EBA is seen in periventricular and perivascular spaces. **B.** In a rat where ICP was increased to 40 mmHg from 30–60 minutes after EBA injection and sacrificed at 2 hours increased EBA penetration from periventricular and peri-ependymal CSF spaces is seen. Cb, cerebellum; cc, corpus callosum; Cp, caudate-putamen; Ctx,

cerebral cortex; FITC, fluorescein isothiocyanate; GFAP, glial fibrillary acidic protein; Hip, hippocampus; LYVE1, lymphocyte vessel endothelial hyaluronan receptor 1; Th, thalamus.

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Table 1.

Physiological variables in rats used for measurement of serum EBA

	Baseline	5 ± 5 min	30 ± 5 min	45 ± 5 min
Control (n = 4)				
ICP (mmHg)	3.8 ± 2.4	3.3 ± 2.6	3.0 ± 1.2	2.3 ± 1.3
MAP (mmHg)	48.3 ± 4.8	48.3 ± 7.1	46.0 ± 5.4	47.5 ± 5.0
Heart rate (beats/min)	301 ± 74 ^a	317 ± 66 ^a	344 ± 84 ^a	351 ± 78 ^a
Elevated ICP (n = 5)				
ICP (mmHg)	4.2 ± 1.4	39.2 ± 0.7 [*]	39.2 ± 0.8 [*]	10.6 ± 2.2 [*]
MAP (BPM)	58.8 ± 8.3	70.0 ± 10.2	75.0 ± 12.1	69.2 ± 8.8
Heart rate (beats/min)	282 ± 43 ^a	307 ± 37 ^a	412 ± 56 ^a	362 ± 91 ^a

^{*} $P < 0.05$ vs. control at same timepoint

^a missing data point(s)