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Comparison Evans Blue Injection Routes: Intravenous vs. Intraperitoneal, for Measurement of Blood-Brain Barrier in a Mice Hemorrhage Model

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Abstract

AIMS—Intracerebral hemorrhage is one of the most devastating subtypes of stroke, leaving survivors with severe neurological deficits. Disruption of the blood brain barrier (BBB) following hemorrhage results in development of vasogenic brain edema, a most life-threatening event after such events as intracerebral hemorrhage (ICH). The Evans Blue assay is a popular method for the quantification of BBB disruption. Although this method is in common use, there are several protocols of the assay in the literature which vary in the route of administration, as well as the circulation time of the stain. In this study, we compared the amounts of accumulated stain in brain tissue following intraperitoneal versus intravenous injection at 0.5, 3 and 24 hours of circulation time.

METHODS—58 CD-1 mice were used. Animals were divided into ICH (N=42), sham groups (N= 6) and naïve (N=10). ICH animals received stereotactic injection of collagenase type VII into the right basal ganglia. Sham animals received only needle trauma. Evans Blue stain was injected 24 hours after collagenase injection or needle trauma. The consistency of ICH produced was characterized by estimation of hematoma volume via hemoglobin assay and neurological evaluation.

RESULTS—The produced hematoma and neurological deficits were well comparable between different experimental groups. There was no statistically significant difference in the results of the Evans Blue assay with regard to administration route.

CONCLUSIONS—The amount of Evans Blue stain accumulated in the brains of mice after ICH produced by collagenase injection was independent of the stain administration route.

Keywords

ICH; BBB; Evans Blue Assay

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INTRODUCTION

The blood-brain barrier (BBB) is a specialized vascular system consisting of endothelial cell tight junctions, basal lamina and glial processes (Rubin and Staddon 1999). It separates circulating blood from cerebrospinal fluid in the central nervous system and has a low permeability to ionized water-soluble molecules with a molecular mass greater than 180 Daltons, unless the molecule can cross into the brain via specific active transporters (Kroll RA and Neuwelt EA 1998). Disruption of the BBB following brain injury results in development of vasogenic brain edema, a most life threatening event after such events as ICH (Xi et al., 2006). Since preservation of the BBB is a common goal among neuroprotective therapies, an objective method for evaluating blood brain barrier disruption is needed.

A common technique of evaluation involves the staining of plasma serum albumin, known as the Evans Blue Assay. The major characteristic of Evans Blue stain is its ability to bind to serum albumin immediately after stain injection into the blood stream (Reeve E.B. 1956). Since plasma albumin does not pass the BBB under normal physiologic conditions, spectrophotometric determination of Evans Blue stain accumulation is an easy and reliable way to estimate BBB permeability. In this study we compared different protocols of this assay and evaluated the difference in the Evans Blue stain accumulation in brain of mice after intraperitoneal versus intravenous injection of dry stain, at three different points of circulation time.

MATERIALS AND METHODS

Animal Groups and Treatment Methods

This study was conducted in accordance with the National Institutes of Health guidelines for the treatment of animals and was approved by the Animal Care and Use Committee at Loma Linda University. Male CD-1 mice (34–43 grams, Charles River, MA, USA) were housed with a 12-hour light/dark cycle with access to water and food *ad libitum*. A total of 58 mice were used. Animals were divided into two groups: a sham (needle-trauma only) group (6 mice) and an ICH group (42 mice). All animals received an injection of Evans Blue dry stain 24 hours after ICH, either intraperitoneally or intravenously. For evaluation of Evans Blue stain in blood, 10 naïve animals were used

Surgical Procedure

The collagenase-induced intracerebral hemorrhage model was used as previously described in mice. Briefly, under general anesthesia [ketamine (100 mg/kg), xylazine (10 mg/kg)] the mice were positioned prone in a stereotaxic frame (Stoelting, Wood Dale, IL, USA), the calvarium was exposed by a midline scalp incision from the nasion to the superior nuchal line, and the skin was retracted laterally. Using a variable speed drill (Fine Scientific Tools, Foster City, CA, USA) a 1.0 mm burr hole was made 0.9 mm posterior to the bregma and 1.45 mm to the right of midline. A 26-gauge needle on a Hamilton syringe was inserted 4.0 mm into the right deep cortex/basal ganglia at a rate 1 mm/min. Collagenase (0.075 units in 0.5 µl saline, VII-S; Sigma, St Louis, MO, USA) was infused into the brain at a rate of 0.25 µl/min over 2 minutes using an infusion pump (Stoelting, Wood Dale, IL, USA). Afterwards, the needle was left in place for an additional 10 minutes following injection to prevent the possible leakage of collagenase solution. Upon removal of the needle, the incision was closed and the mice were allowed to recover. Sham operation was performed with needle insertion only.

Evaluation of BBB Permeability

A 2% solution of Evans Blue in normal saline (4 mL/kg of body weight) was injected either into the jugular vein or intraperitoneally. The stain was allowed to circulate for 30 minutes, 3 or 24 hours. In sham-operated animals, intravenous injection of Evans Blue was done. Stain was allowed to circulate for 24 hours.

Afterwards, the mice were then transcardially perfused with 50ml of ice-cold PBS, the brain tissue was removed and divided into right and left hemispheres, frozen in liquid nitrogen, and stored at -80°C . Right hemisphere samples were homogenized in 1100 μL of PBS, sonicated and centrifuged (30 min, 15000 rcf, 4°C). The supernatant was collected in aliquots. To each 500 μL aliquot an equal amount of 50% trichloroacetic acid was added, incubated over night by 4°C and this was then centrifuged (30 min, 15000 rcf, 4°C). Evans Blue stain was measured by spectrophotometer (Thermo Spectronic Genesys 10 uv, Thermo Fischer Scientific Inc., Waltham, MA, USA). at 610 nm and quantified according to a standard curve. The results are presented as (μg of Evans Blue stain)/(g of tissue).

Quantification of Evans Blue stain in blood

Concentration of Evans blue in blood plasma was evaluated as described before (Xu et al., 2001) Briefly 2% solution of Evans Blue in normal saline (4 mL/kg of body weight) was injected either into the jugular vein or intraperitoneally. Stain was allowed to circulate for 3 hours. After final anesthesia, blood samples were collected by cardiac puncture in a heparinized syringe. These blood samples were centrifuged at 3 000 rpm for 5 minutes and diluted to 1/50 of the original concentration. Blood plasma was collected. To each 75 μL aliquot an equal amount of 50% trichloroacetic acid was added, incubated overnight at 4°C and then centrifuged (30 min, 15000 rcf, 4°C). Evans Blue stain was measured by spectrophotometer (Thermo Spectronic Genesys 10 uv, Thermo Fischer Scientific Inc., Waltham, MA, USA). at 610 nm and quantified according to a standard curve.

Evaluation of hematoma value (hemoglobin assay)

Initially, a standard curve was obtained using a "virtual" model of hemorrhage. Hemispheric brain tissue was obtained from naive mice subjected to complete transcardial perfusion to remove intravascular blood. Incremental volumes of homologous blood (0, 2, 4, 8, 16, 32 μL) were added to each brain tissue sample with phosphate buffered saline (PBS) to reach a total volume of 1100 μL , followed by homogenization for 30 sec, sonication on ice for 1 min, and centrifugation at 15,000 rpm for 30 min. Drabkin's reagent (0.8 ml, Sigma) was added to 0.2 ml supernatant aliquots and allowed to stand for 15 min at room temperature. Optical density was measured and recorded at 540 nm with a spectrophotometer (Thermo Spectronic Genesys 10 uv, Thermo Fischer Scientific Inc., Waltham, MA, USA). These procedures yielded a linear relationship between measured hemoglobin concentrations in perfused brain and the volume of added blood.

For hematoma evaluation, supernatant collection as described above (see evaluation of BBB permeability) was used. Similar to the procedure used for standard curve generation, Drabkin's reagent (0.8 ml, Sigma) was added to 0.2 ml supernatant aliquots and allowed to stand for 15 min at room temperature. Optical density was measured and recorded at 540 nm with a spectrophotometer (Thermo Spectronic Genesys 10 uv, Thermo Fischer Scientific Inc., Waltham, MA, USA). *Neurobehavioral Evaluation.* Mice were evaluated by tester blinded to the animal groups. Three tests were implemented for evaluation of neurological deficits: 1) Modified Garcia test, in which mice were given a score of 0 to 21 (normal). The scoring system consisted of 7 tests, (spontaneous activity, axial sensation, vibrissae proprioception, limb outstretching, lateral turning, forelimb walking and climbing) with a possible score range of 0 to 3 points (0=worst; 3=best), a minimum total score of 0 and a

maximum of 21 points; 2) Wire hanging test; and 3) Beam balance test. The latter two tests utilized bridges (550cm wire or 590cm beam) between two platforms on which the subjects were placed in the center and then evaluated according to six criteria based on each subject's ability to reach the platform and/or use its limbs in a symmetrical manner, for which they were assigned a score of 0 to 5 points (normal). The average of three trials per test for each animal was calculated.

Statistical Analysis

Quantitative data were expressed as mean $\pm$ SEM. Statistical significance was verified by analysis of variance/ANOVA (Turkey test). Significance was accepted at $p < 0.05$.

RESULTS

No neurological deficit between ICH animals in different groups was observed

Results of neurological testing are summarized in Fig 1 (A, B, C). All animals that received collagenase injections demonstrated significant neurological deficit ($p < 0.05$ vs. Sham). No statistically significant difference between experimental groups was observed.

Evans Blue Stain did not affect results of hemoglobin assay

There was no noted difference in the macroscopically evaluated hemorrhage sites between ICH animals and ICH animals after Evans Blue stain injection (Fig. 2A and Fig. 2B). Evans Blue accumulation in the contralateral hemisphere was visible macroscopically (Fig. 2B, anterior view, black arrow). However, no difference in the results of the hemoglobin assay between ICH animals and ICH animals after Evans Blue stain injection was observed (Fig. 2C). The average hematoma volumes in animals that did not receive Evans Blue stain was calculated to be 100%.

Collagenase injection caused comparable bleeding in all ICH animals

No statistically significant difference in hematoma volumes between animals in all ICH groups was observed. Hematoma volumes were evaluated microscopically (Fig. 2B) and measured by hemoglobin assay (Fig. 3).

The amount of Evans Blue stain in mouse blood 3 hours after stain injection was independent of the route of administration

Comparing intraperitoneal and intravenous administration routes we found that there was no statistical difference in the amount of Evans Blue stain remaining in the blood of mice after 3 hours of circulation time (data not shown)

The amount of Evans Blue stain accumulated in mouse brains after ICH was independent of the route of administration

With an increase in circulation time from 30 min (Fig. 4A) to 24 hours (Fig. 4B) a considerable time-dependent stain accumulation in sclera and pinnae of animals was observed. Well in agreement with this finding we observed an increase in accumulated stain in the brain (Fig. 4C) (The results are presented as (μg of stain)/(g of tissue)).

However, at all time points there was no statistical difference in accumulation for intraperitoneal versus intravenous routes of stain administration. We did note extravasation of stain along the needle track in the sham-operated animals (4D).

DISCUSSION

The aim of this study was to establish whether the administration route or circulation time would affect the amount of Evans Blue delivered across the blood-brain-barrier into the brain of mice after ICH.

Brain edema is most devastating life-threatening complication after ICH. Multiple forms of edema are present after ICH, but the main form is likely vasogenic (Xi et al. 2006, Adeoye et al., 2010) in origin. Several mechanisms have been proposed to be responsible for the development of peri-hematoma edema. The increased permeability of the blood-brain-barrier plays the greatest role in the development of vasogenic brain edema.

For evaluation of BBB-permeability, several methods exist. Because other methods such as magnetic resonance imaging (MRI) (Floris et al, 2004 MacLellan et al., 2008) utilize radioactive tracers (Preston et al., 2002) are expensive and present technical challenges for handling and administration, Evans Blue assay remains the most popular methods for evaluation of BBB-disruption in experimental studies of brain injury. Although this assay is widely used in numerous publications, it has not been previously standardized. One can find in the literature two different descriptions of administration route: intraperitoneal (Morrey et al., 2008) and intravenous (Belaev et al., 2005) injection. There exists a wide variation in the reported times of stain circulation. Mychaskiw et al. and Kolzler et al. allowed the stain to circulate for 20 minutes (Mychaskiw et al., 2006, Kolzler 2003). Hellal et al. used a one hour circulation period (Hellal et al., 2004), while Belaev et al. used 2 hours (Belaev 2005) and Kim et al. described using a 24 hour stain circulation time (Kim et al., 2009).

In our study, we compared the amounts of accumulated Evans Blue in the brain after intraperitoneal and intravenous injection at three different time-points. To test our hypothesis we used the collagenase model of ICH induction. The model was originally described by Rosenberg (Rosenberg et al. 1990) and adapted for use in the mouse model by Tang et al. (Tang et al., 2004) and used by Wu et al. (Wu et al., 2010). The greatest advantage of this method is that it provides a model for the spontaneous bleeding and hematoma enlargement that occurs in 30% of patients (Kazui et al., 2006). It is however understood that the amount of bleeding caused by collagenase injection and resulting brain edema in this model are not highly reproducible. Consistency of the model was characterized via neurological testing and hemoglobin assay. Since several studies have shown that the degree of brain edema surrounding the hematoma is associated with poor neurological outcome (Zazulia et al., 1999), we evaluated post-ICH neurological deficits to confirm consistency of our model by doing three different neurological tests as we have done previously (Manaenko et al., 2009). Modified Garcia Scoring, Wire Hanging and Beam Balance tests were employed. All animals after collagenase injection developed neurological deficits. There were no statistically significant differences between the experimental groups. Hemorrhage volume produced by collagenase injection was investigated by evaluation of the amount hemoglobin deposited in brain tissue. For this propose we used a spectrophotometric hemoglobin assay originally described by Choudhri et al. for the objective quantification of intracerebral hemorrhage in mice (Choudhri et al., 1997). In this method, total hemoglobin is rapidly converted to cyanmethemoglobin at alkaline pH levels (Drabkin et al 1935) and the absorbance of the derivative is determined at 540 nm. Even though there is a difference between the adsorption peak of hemoglobin cyanderivative (540 nm) and the adsorption used for Evans Blue assay (610 nm), we still performed the test to see if Evans Blue stain could affect the results of the hemoglobin assay. For this, ICH was induced in 6 animals. Three animals received intravenous injection of Evans Blue stain, which was allowed to circulate for 24 hours. Results of the hemoglobin assay were then compared with animals that did not receive stain injection. Since no

difference was determined we concluded that the stain did not affect the hemoglobin evaluation.

Moreover, further experiments demonstrated that there were no significant differences in hematoma volumes between different experimental groups. The amount of blood caused by injection of collagenase in our study was well in agreement with previous publications, in which authors used similar methods of ICH induction (Foerch et al., 2009; Kim et al., 2009). Furthermore we found out that after 3 hours of circulation time there was no route-dependent difference in Evans Blue stain concentration in the blood of naïve animals. After characterization of model consistency we compared accumulation of stain in the brains of sham (24 hours circulation time) versus ICH animals after 0.5, 3 and 24 hours of stain circulation following intraperitoneal or intravenous stain administration. Slight accumulation of the stain was observed in brains of sham-operated animals along the needle track (which we surmised to be due to mechanical needle trauma). In our study, the measured amount of Evans Blue stain accumulated in the brains of animals after ICH is well in agreement with other studies. (MacLellan et al., 2006, Tang et al., 2010, Manaenko et al., 2010). We observed a strong time-dependent tendency towards increase in stain accumulation by both administration routes. In none of the time points was there observed a difference between intraperitoneal and intravenous administration.

CONCLUSION

Evans Blue stain accumulation in the brains of mice after ICH is independent from administration route and circulation time.

Research highlight

Mouse models are more and more popular in recent years, due to the advantages of transgenic mice species. However, the small size of mouse introduced technical challenges such as intravenous injection. One of the brain physiological studies is the study of the blood-brain barrier (BBB) and one of the most popular methods of BBB evaluation is by Evans Blue measurement. In rat models, Evans Blue is injected intravenously. However, intravenous injection in mouse is difficult. Therefore, intraperitoneal injection Evans Blue will be an extremely convenient method. But no study had been done to compare if intraperitoneal injection will be comparable with intravenous injection.

This is the first study that compared intravenous and intraperitoneal injection routes of Evans Blue in a mouse model. We found the Evans Blue contents are similar by these two routes.

Second, these two methods are tested in an intracerebral hemorrhage mouse model and achieved similar results.

Therefore, this new study suggests that intraperitoneal injection of Evans Blue is comparable with intravenous injection and is an easy, practical, and accurate method of BBB evaluation.

LIST OF ABBREVIATIONS

ICH	Intracerebral hemorrhage
BBB	Blood-brain-barrier

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Figure 1. Neurological evaluation

Significant neurological deficits were observed in all collagenase-injected animals.

(* $p < 0.001$ vs sham)

No differences between experimental groups were detected.

(Sham $N = 6$;

i.p. 30 min circulation time $N = 6$; 3 hrs circulation time $N = 6$;

24 hrs circulation time $N = 6$

i.v. 30 min circulation time $N = 6$; 3 hrs circulation time $N = 6$;

24 hrs circulation time $N = 6$

Figure 2. Comparison of hemoglobin assay results between animals with and without Evans Blue stain injection

The Evans Blue stain, which is well-visible macroscopically, did not affect results of the hemoglobin assay. An average hematoma volume in the experimental group without stain injection was determined to be 100%. There was no significant difference between animals without (N = 3) and with stain (N = 3) injection.

Figure 3. Hemorrhage volume evaluation

Collagenase injection produced bleeding without significant difference between experimental groups (30 minutes of circulation time $p=0.673$; 3 hours of circulation time $p=0.660$; 24 hours of circulation time $p=0.105$).

Figure 4. Evans Blue Assay

Increase of Evans Blue stain accumulation in sclera and pinnae of animals after 24 hours stain circulation following i.v. injection (Fig. 4B, black arrows) compared with 30 stain circulation time (Fig. 4A). However, there were no statistical differences between the two routes of stain administration at any time point. Strong tendency by 24 hours of circulation time did not reach significance ($p = 0.097$). Slight stain accumulation was observed in the brains of sham operated animals (Fig. 4, black arrow heads).