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Comparative Analysis of H&E and Prussian Blue Staining in a Mouse Model of Cerebral Microbleeds

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Summary

Cerebral microbleeds are microscopic hemorrhages with deposits of blood products in the brain, which can be visualized with MRI and are implicated in cerebrovascular diseases. Hematoxylin and eosin (H&E) and Perl's Prussian blue are popular staining methods used to localize cerebral microbleeds in pathology. This paper compared these two staining techniques in a mouse model of cerebral microbleeds. We used lipopolysaccharide (LPS) to induce cerebral microhemorrhages. C57B6 mice were treated with LPS (5 mg/kg, i.p.) or vehicle at baseline and at 24 hr. The brains were extracted 48 hr after the first injection and adjacent coronal sections were stained with H&E and Prussian blue to compare the effectiveness of the two staining techniques. H&E-positive stains were increased with LPS treatment and were correlated with grossly visible microhemorrhages on the brain surface; Prussian blue-positive stains, by comparison, showed no significant increase with LPS treatment and did not correlate with either H&E-positive stains or surface microhemorrhages. H&E staining is thus a more reliable indicator of acute bleeding events induced by LPS in this model within a short time span. (J Histochem Cytochem 62:767–773, 2014)

Keywords

Cerebral microbleeds, H&E, Prussian Blue, LPS

Introduction

Cerebral microbleeds ("microbleeds" or "microhemorrhages") are small hemorrhages with deposits of blood products in the brain, which can be visualized with MRI. Microbleeds increase with age (Poels et al. 2010) and in diseases such as cerebral amyloid angiopathy (CAA), hypertension, stroke, and vascular dementia (Vernooij et al. 2008; Cordonnier and van der Flier 2011), and are associated with intracerebral hemorrhage (ICH), cognitive decline (Lovelock et al. 2010; Qiu et al. 2010; Bokura et al. 2011; Poels et al. 2012) and platelet therapies (Lee et al. 2009; Vernooij et al. 2009; Lovelock et al. 2010; Ge et al. 2011).

Whereas microbleeds are observed clinically on MRI, they are identified by hematoxylin and eosin (H&E) and Perl's Prussian blue stains in neuropathology studies (Cullen et al. 2005; Fisher et al, 2010). Both staining techniques are frequently used and reveal areas within the tissue that have been exposed to blood, but they each rely on different mechanisms for staining. In H&E, hematoxylin stains nucleic acids whereas eosin counter-staining is used to detect the cytoplasm. Red blood cells (RBCs) stain a distinguishable red-orange under H&E. Prussian blue, on the other hand, detects bleeding in an indirect manner by staining for ferric iron, which is formed after the lysis of RBCs.

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When ferric iron storage complexes are exposed to ferrocyanide, the reaction produces insoluble Prussian blue dye. Given that Prussian blue staining is relatively insensitive for localizing iron within brain tissue, trace amounts of iron within the blood plasma are likely insufficient to produce localizable insoluble blue crystals (Perl and Good 1992).

In a previous model of cerebral microbleeds using aged Tg2576 mice, diseased blood vessels could be successfully visualized with Prussian blue staining (Vasilevko et al. 2010; Fisher et al. 2011). In another mouse model of cerebral microbleeds, both H&E and Prussian blue showed comparable results 3 to 4 days post intracranial injection of β-amyloid (Aβ) antibody in APPswe/PS1d9 mice (Meyer-Luehmann et al. 2011). In the present study, based on a previously published model of blood brain-barrier (BBB) permeability alteration (Xaio et al. 2001; Veszelka et al. 2003), we used an even shorter time window (2 days) for cerebral microbleed induction and compared the results obtained with H&E and Prussian blue stains.

Materials & Methods

Animal Studies

All animal procedures were approved by UCI Institutional Animal Care and Use Committee and were carried out in compliance with University Laboratory Animal Resources regulations. We used lipopolysaccharide (LPS) derived from *Salmonella* Typhimurium (Sigma-Aldrich, St. Louis, MO) to induce microbleeds in mice aged 3–4 months. Mice (C57B6 background) were purchased from Taconic (Taconic, Hudson, NY). In one set of experiments, we measured BBB permeability to sodium fluorescein (NaF). Mice were injected with LPS (i.p., 5 mg/kg) or equal volume of vehicle (phosphate-buffered saline, PBS) at 0 and 24 hr (n=5 and 6, respectively). After 48 hr of PBS or LPS treatment, 100 μL sodium fluorescein (Sigma-Aldrich , St. Louis, MO) (12 mg/ml) was injected intravenously. After 1 hr, mice were anesthetized and perfused with cold PBS, as follows. Briefly, the animals were anesthetized by administering a lethal dose of Nembutal (150 mg/kg, i.p.). The level of anesthesia was monitored by an absence of response to toe pinching. Once the animal was deeply anesthetized, a surgical incision was made and the chest cavity was opened. The thoracic cage was reflected exposing the heart. A 21–25-gauge needle was used as a cannula and inserted into the left ventricle and cold PBS solution was applied at a flow rate of 7–8 ml/min. After perfusion for 5 min, brains were isolated, weighed, and homogenized in 1 ml PBS. The homogenized brain tissue was centrifuged at 1,000 × g for 15 min at 4°C. Using a serial dilution of a NaF solution, a standard curve was prepared and the unknown concentration of NaF in the brain homogenate supernatant was determined by a fluorescence multiwell plate reader, using a fluorescent filter pair [Ex(k) 485 ± 10 nm; Em(k) 530 ± 12.5 nm]. In another set of experiments, mice were injected with LPS (i.p., 5 mg/kg) or an equal volume of vehicle (PBS) (n=8 and 11, respectively) at 0 and 24 hr. Forty-eight hr after the initial injection, cardiac perfusion with ice-cold PBS was performed for 5 min and the brains were isolated and fixed in 4% paraformaldehyde to study cerebral microhemorrhage development.

Detection of Microhemorrhages

Microhemorrhages on the brain surface were observed by two blinded, independent observers and the average of the two used for analysis (n=8 for PBS; n=11 for LPS). After observation of surface hemorrhages, brains were sectioned with a vibratome and coronal sections (40 μm) of mouse brains were collected (brains damaged during removal were not sectioned). Every 6th section and a total of 16 sections per brain were selected for H&E staining. Adjacent sections of those used for H&E staining were used for Prussian blue staining. Prussian blue staining was performed using freshly prepared 5% potassium hexacyanoferratetrihydrate and 5% hydrochloric acid. Thirty min later, sections were rinsed in water and counterstained with nuclear fast red, dehydrated, and covered. H&E staining was performed by Research Services Core offered by the Department of Pathology and Laboratory Medicine at the UCI Medical Center. Microbleeds were identified at 20× magnification as a collection of RBCs that appear red-orange using H&E staining and as clear purple blue deposits using Prussian blue staining. Images of the observed positively stained sections were taken using an Olympus BX40 microscope and CC-12 Soft-Imaging System with Olympus MicroSuite (TM)-B3SV software (Olympus, Tokyo, Japan).

Data Analysis

Data are presented as mean ± SEM, and all statistical analyses were performed using Microsoft Excel 2007 (Redmond, WA). Differences in the mean between two groups were tested using the Student's t-test. A p-value of less than 0.05 was considered statistically significant.

Results

LPS-treated mice (n=11) had grossly visible microhemorrhages on the brain surface, whereas control animals receiving PBS (n=8) had negligible microhemorrhages (Fig. 1A). LPS treatment induced cerebral microhemorrhages and the number of microhemorrhages was significantly higher in the LPS-treated animals compared with vehicle-treated control animals (30.2 ± 7.4 vs 0.25 ± 0.16, p<0.001) (Fig. 1B). LPS increased BBB permeability to sodium fluorescein (NaF) by 2.8-fold, from 2.9 ± 0.3 mg NaF per gram of brain tissue in the control group to 8.2 ± 1.8 mg NaF per gram of

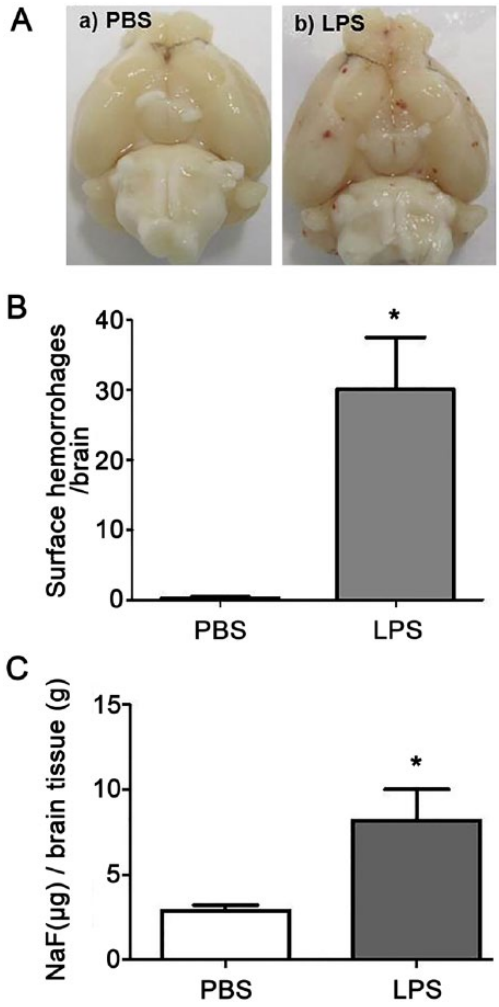

Figure 1. (A) Representative images of isolated brains from (a) control (vehicle-treated) and (b) LPS-treated mice. (B) LPS significantly increased microscopic hemorrhages on the brain surfaces. Values represent mean ± SEM (PBS-treated group, n=8; LPS-treated group, n=11; p<0.05). The results were determined by two independent, blinded observers. (C) LPS significantly increased brain permeability to sodium fluorescein extravasation (vehicle-treated group, n=5, LPS-treated group; n=6; p<0.05).

brain tissue in the LPS-treated group (n=6 and 5, respectively, p<0.05) (Fig. 1C).

LPS induced fresh hemorrhages in brain tissue, as revealed by H&E staining, whereas control mice receiving PBS had negligible positive staining (Fig. 2A). In mice treated with LPS (n=9), the average number of microscopic hemorrhages was 2.1 ± 0.8 per brain section (p<0.001 vs control, n=6) (Fig. 2B). The number of microbleeds found in the brain parenchyma by H&E staining correlated highly with the number found on the brain surface (R = 0.87, p<0.01) (Fig. 2C). Microbleeds appeared to be distributed comparably in neocortical (1.1 ± 0.4 per brain section) and non-neocortical regions (1.0 ± 0.4 per brain section), with non-neocortical regions including the hippocampus, striatum, thalamus, and hypothalamus (Fig. 2D). Prussian blue-positive stains were found in both vehicle- and LPS-treated groups (Fig. 3A). The difference in the average number of Prussian blue-positive stains per section between the LPS- and vehicle-treated groups was not statistically significant (Fig. 3B). Prussian blue-positive stains did not correlate with surface hemorrhages (Fig. 3C). Further, a comparison between H&E and Prussian blue staining suggested that there was no correlation between the positive stains (Fig.4A–4G). However, signs of hemorrhaging were observed occasionally on Prussian blue-stained sections (Fig. 4B, 4D, 4F) as brown (indigenous color of RBCs, not as a result of Prussian blue staining), which often coincided with H&E-positive stains (Fig. 4A, 4C, 4E).

Discussion

In this study, we compared H&E staining with Prussian blue staining in a non-invasive model of cerebral microbleeds that showed the rapid production of cerebral microscopic hemorrhages. The number of H&E-positive lesions was significantly higher in LPS-treated mice as compared with control, vehicle-treated mice, and the results correlated with brain surface microhemorrhages. On the other hand, Prussian blue-positive staining was not significantly higher in LPS-treated mice and did not correlate with either H&E-positive staining or surface microhemorrhaging.

Prussian blue staining has been used previously to visualize iron deposition near diseased blood vessels in transgenic mice (e.g., Tg-2576) that spontaneously over-express β-amyloid (Vasilevko et al. 2010; Fisher et al. 2011). Because vessel leakiness increases over time in these mice (it may take two years for the transgenic animals to fully show microbleeds), the use of Prussian blue was justified (Vasilevko et al. 2010). In another study, 3 to 4 days were sufficient for the use of Prussian blue, as evident from the comparable H&E- and Prussian blue-stained microbleed frequency profiles (Meyer-Luehmann et al. 2011). In the same study, H&E- and Prussian blue-positive staining was observed even in control animals. In the current study, however, H&E produced negligible positive staining in the control group. This could be due to cardiac perfusion performed in the present, but not in the previous, study. On the other hand, Prussian blue staining was positive in the controls in the present study, which is generally consistent with previous findings (Meyer-Luehmann et al. 2011). As Prussian blue stains for iron deposits that may come from sources other than bleeding, the localized Prussian blue-positive foci within the control group may not be microbleeds. The Prussian blue-positive stains may also be from pre-existing bleeding that was not induced by LPS treatment. In addition, when comparing adjacent sections, Prussian blue staining

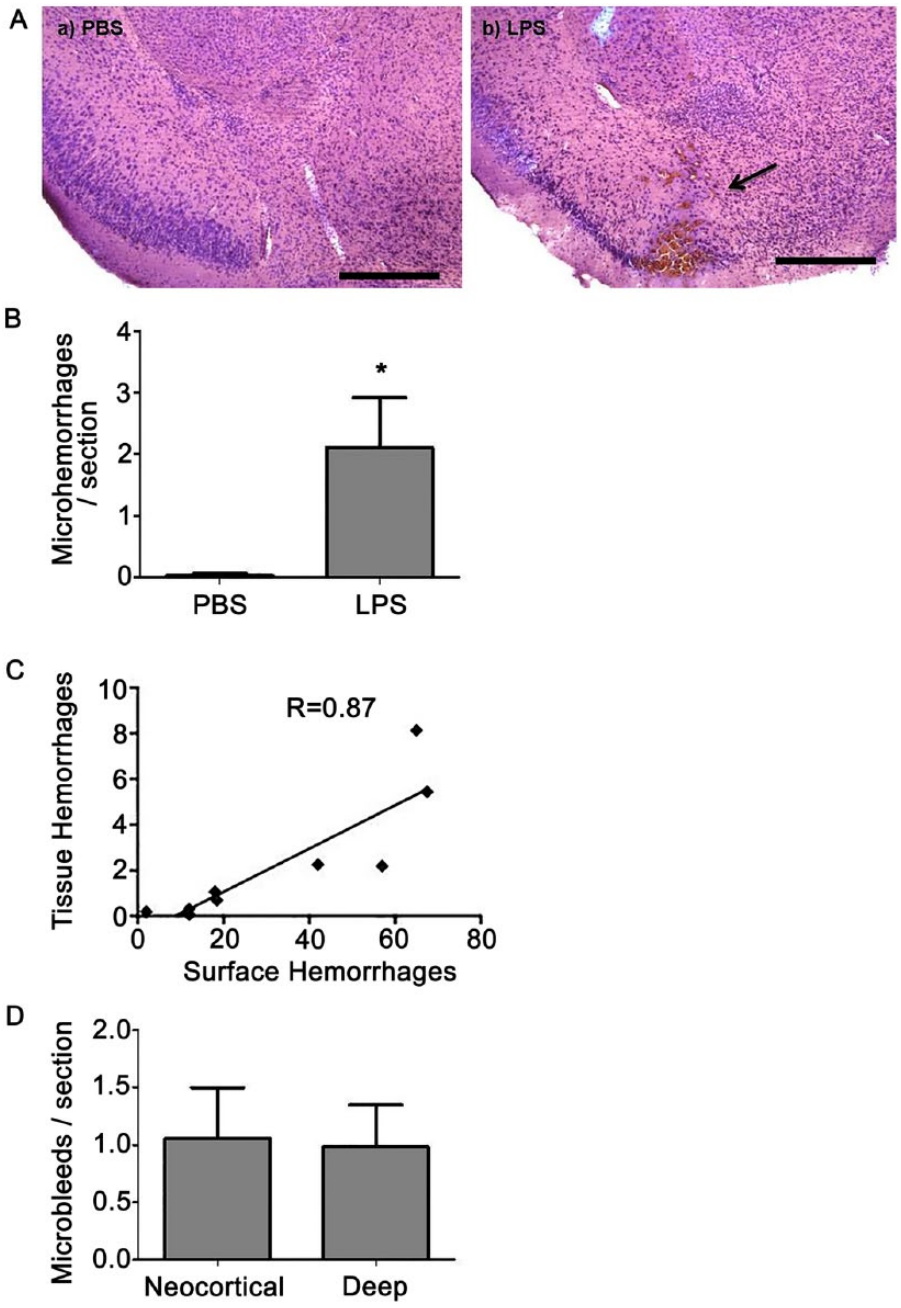

Figure 2. (A) H&E staining: representative brain sections of (a) control (vehicle-treated) and (b) LPS-treated mice. Scale bar, 500 µm, 4× magnification. (B) LPS significantly increased the average number of microscopic hemorrhages in brain tissue (*p<0.001). (C) Surface and H&E-positive (tissue) staining (for parenchymal microhemorrhages) were highly correlated (n=9, R = 0.87, p<0.01). (D) The mean number of microbleeds per section did not differ between neocortical and deep regions. Neocortical region refers to cerebral neocortex; deep region includes hippocampus, striatum, thalamus, and hypothalamus. Values represent the mean ± SEM.

was negative where RBC remnants were clearly visible, and the corresponding area on adjacent sections stained positively with H&E staining. Given that Prussian blue often stains positive in control tissues but fails to detect apparent bleeding events in treated animals, it is not surprising that LPS-treated animals did not have a significantly higher degree of Prussian blue-positive staining than that in the control animals.

Prussian blue detects old hemorrhaging, and closer inspection of the Prussian blue mechanism provides a possible explanation for this phenomenon. Following a hemorrhage in the brain, RBCs survive for at least several days in the tissue, from anywhere between 2 to 10 days (Wagner et al. 2003), and these cells lyse once intracellular energy reserves deplete (Hua et al. 2007). RBC lysis occurs slowly with the release of oxyhemoglobin, which is converted to deoxyhemoglobin. Deoxyhemoglobin is then spontaneously and non-enzymatically oxidized to methemoglobin as ferrous iron is converted to ferric iron (Bradley 1993). Heme-released iron occurs first as iron-ferritin and later as ferric iron rich-hemosiderin.

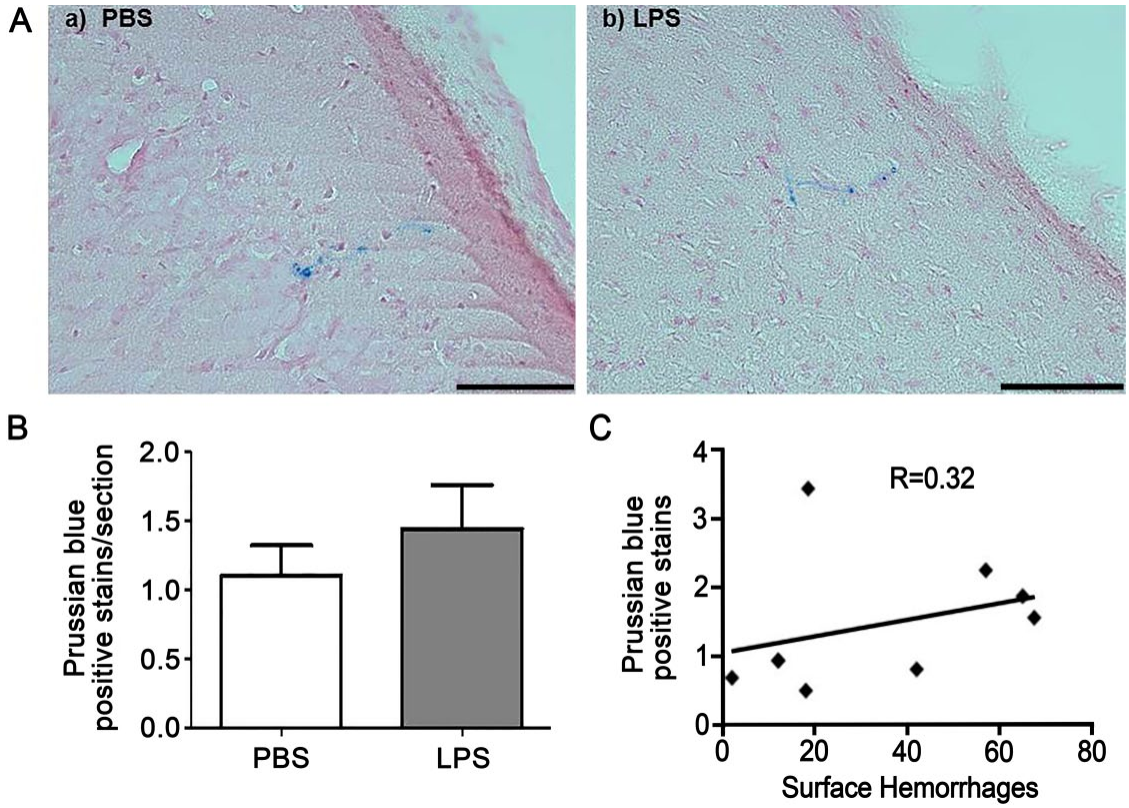

Figure 3. (A) Prussian blue staining: representative brain sections of (a) control (vehicle-treated) and (b) LPS-treated mice. Scale bar, 100 µm, 20× magnification. (B) Average number of Prussian blue-positive stains did not differ between the experimental groups. (C) Prussian blue-positive stains (per section) and surface hemorrhages were not significantly correlated (n=9 with two points overlapped, R = 0.32, p=0.40). Values represent mean ± SEM (vehicle-treated group, n=6; LPS-treated group, n=9). Results were read by a blinded observer.

Hemosiderin is an inert molecule that is not removed from the brain and hence is used as a marker of an old hemorrhage (Wagner et al. 2003). In the Prussian blue reaction, treatment with a dilute hydrochloric acid solution unmasks ferric iron bound to ferritin. This ferric iron then reacts with a dilute solution of potassium ferrocyanide to produce a blue insoluble compound known as Prussian blue. The ferrous ions themselves have no colored product and therefore their reaction cannot be seen. This entire process of degradation of hemoglobin to heme to the release of iron requires several days. Previous studies have shown that hemosiderin first appears near the lesion after approximately 6 days in humans and 5 days in a rabbit ICH model (Wagner et al. 2003) and did not become obvious in histochemical staining for 5–7 days after traumatic brain injury (Gilliland et al. 1991). In the present study, bleeding may not occur immediately after LPS injection and the bleeding observed in this study may be less than 2 days old. Thus, there may not be sufficient time for iron to be released from lysed RBCs for detection by Prussian blue.

The model used in this study is adapted from a previously published model of BBB permeability alteration. According to Veszelka et al. (2003), petechiae were observed in the cerebellum and midbrain of animals injected thrice with LPS at 3 mg/kg. We used a comparable injection scheme (two injections of LPS at 5 mg/kg), which induced the rapid production of cerebral microscopic hemorrhage visible grossly and microscopically. The rationale for using multiple injections is to decrease variability, as reported in previous studies on BBB permeability (Xaio et al. 2001; Veszelka et al. 2003). Because LPS is known to cause BBB breakdown (Diamond et al. 2009) and induce apoptosis in brain endothelial cells (Munshi et al. 2002; Karahashi et al. 2009), it is likely that the erythrocyte extravasation observed in the current study may be the consequence of disrupted BBB and/or endothelial apoptosis.

In summary, Prussian blue-positive stains were detected in controls and only increased slightly with LPS treatment. Prussian blue did not react with fresh bleeding and did not correlate with either surface hemorrhages or parenchymal

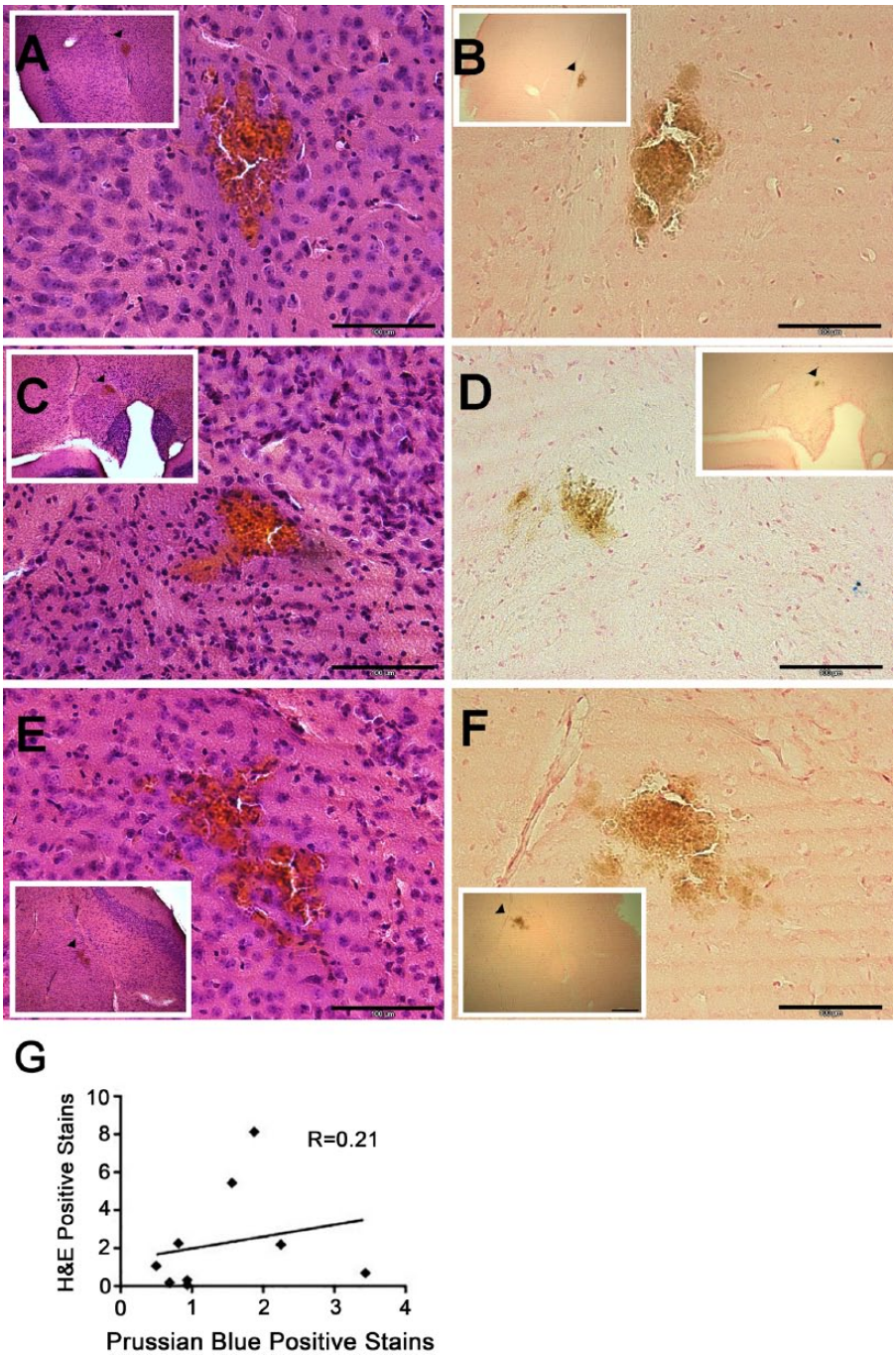

Figure 4. (A–F) H&E (A, C, E) and Prussian blue (B, D, F) staining was performed on adjacent sections. In the H&E-positive stained sections, red represents red blood cells (RBCs, black arrowhead). Occasionally, RBCs can be seen on Prussian blue stained sections as brown (black arrowhead), which often coincides with the red staining in the H&E-stained sections. Brown is the indigenous color of red cells, not indicative of a Prussian blue-positive stain. Scale bar, 100 μm, 20× magnification. Inlet: 4× magnification; images show the location of the microbleed. (G) There is no significant correlation between the total number of Prussian blue-positive stains and H&E-positive stains (n=9, R = 0.21, p= 0.58).

microhemorrhages, as detected by H&E staining. The positive stains observed by Prussian blue are likely pre-existing microhemorrhages or iron deposits from other sources. Therefore, Prussian blue is a less reliable indicator of microhemorrhages than H&E in this model due to a) unclear source of iron in the control tissues, and b) its inability to react with fresh bleeds. Because H&E is only effective at examining fresh hemorrhages, and Prussian blue is only effective in detecting old hemorrhages, future models of microbleeds should be designed with a time frame optimized for use of the respective staining techniques.

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Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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