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Evaluation of the hematoma consequences, neurobehavioral profiles, and histopathology in a rat model of pontine hemorrhage:

Laboratory investigation

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Abstract

Object—Primary pontine hemorrhage (PPH) represents approximately 7% of all intracerebral hemorrhages (ICHs) and is a clinical condition of which little is known. The aim of this study was to characterize the early brain injury, neurobehavioral outcome, and long-term histopathology in a novel preclinical rat model of PPH.

Methods—The authors stereotactically infused collagenase (Type VII) into the ventral pontine tegmentum of the rats, in accordance with the most commonly affected clinical region. Measures of cerebrovascular permeability (brain water content, hemoglobin assay, Evans blue, collagen Type IV, ZO-1, and MMP-2 and MMP-9) and neurological deficit were quantified at 24 hours postinfusion (Experiment 1). Functional outcome was measured over a 30-day period using a vertebrobasilar scale (the modified Voetsch score), open field, wire suspension, beam balance, and inclined-plane tests (Experiment 2). Neurocognitive ability was determined at Week 3 using the rotarod (motor learning), T-maze (working memory), and water maze (spatial learning and memory) (Experiment 3), followed by histopathological analysis 1 week later (Experiment 4).

Results—Stereotactic collagenase infusion caused dose-dependent elevations in hematoma volume, brain edema, neurological deficit, and blood-brain barrier rupture, while physiological variables remained stable. Functional outcomes mostly normalized by Week 3, whereas

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Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

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neurocognitive deficits paralleled the cystic cavitary lesion at 30 days. Obstructive hydrocephalus did not develop despite a clinically relevant 30-day mortality rate (approximately 54%).

Conclusions—These results suggest that the model can mimic several translational aspects of pontine hemorrhage in humans and can be used in the evaluation of potential preclinical therapeutic interventions.

Keywords

pontine hemorrhage; neurobehavioral testing; histopathology; rat; vascular disorders

Primary pontine hemorrhage is a subtype of ICH resulting from the spontaneous rupture of perforating (paramedian) basilar artery branches into the ventral pontine tegmental junction with the basis pontis.[52,117,138] This devastating injury has a yearly incidence of 1 per 50,000 people, accounting for approximately 140,000 (approximately 7%) of 2 million worldwide ICHs[32,102] and having a 65% mortality rate despite contemporary medical-surgical approaches.[31,80,137]

Uncontrolled hematoma expansion worsens patient outcomes after pontine hemorrhage.[3,8,26,39,85,128] In addition, approximately half of the survivors will acquire lesions that cause lasting cognitive deficits in motor learning and visuospatial ability.[74,75,88] Greater disability occurs after larger and bilaterally extending hematomas.[10,15,60,98] This deterioration, however, is poorly understood[102] and is a clinical burden that underscores the need for further translational work.[56,87]

Collagenase-induced ICH in rodents is a useful tool for therapeutic investigations of hemostasis, neurobehavior, and histopathology outcomes.[1,33,71,105,121] Our preliminary report showed the feasibility of pontine hemorrhage in this animal model, using an established pontine infusion approach[66,91] modified by the collagenase ICH modeling technique.[105] This is shown to avoid the limitation of intraventricular bleeding found after experimental autologous blood infusion into the brainstem.[16] In the present study, therefore, we hypothesized that this rodent model of collagenase infusion can mimic outcomes after spontaneous pontine hemorrhage in humans, for use in the further evaluation of therapeutic interventions.[3,10,26,39,60,75,98,114,128]

Methods

Animals and Operative Procedure

In this present study we used 109 adult male Sprague-Dawley rats (Harlan) weighing 290-345 g. All procedures were in full compliance with the *Guide for the Care and Use of Laboratory Animals* (NIH publication no. 85-23) and were approved by the Institutional Animal Care and Use Committee at Loma Linda University. Surgeries involved isoflurane anesthesia (4% induction, 2% maintenance, 70% medical air, and 30% O_2), and all procedures included standard aseptic techniques. The anesthetized animals were secured prone onto a stereotactic frame (Kopf Instruments) and an incision was then made over the scalp. The following stereotactic coordinates were measured over the exposed cranium (caudally, laterally, and to a depth) from the bregma to localize the right ventral pontine tegmentum: 10.2 mm (caudal), 1.4 mm (lateral), and 9.15 mm (deep). Using a standard dental drill, a 1-mm borehole was then formed, through which a 27-gauge needle was inserted, and collagenase Type VII-S (0.2 U/μl; Sigma) was infused by microinfusion pump (Harvard Apparatus) at a rate of 0.2 μl/minute. The syringe remained in place for 10 minutes to prevent back-leakage before being slowly withdrawn (1 mm/minute). A thermostat-controlled heating blanket maintained the core temperature (37.0°C ± 0.5°C)

throughout the entire operation. Control surgeries consisted of needle insertion alone. The borehole was then sealed with bone wax, the incision was sutured closed, and the animals were followed during the recovery period. Food, water, and postoperative supportive care were provided, as is routinely done.[42] Dose-dependent infusions (0.15 and 0.3 U collagenase) were used in the short-term 24-hour study (that is, Experiment 1 [hemorrhagic volume, brain water content, neuroscore, physiological variables {Table 1}, and vascular permeability, Western blot]). Thereafter, infusions of 0.15 U collagenase were evaluated in the long-term 30-day outcome studies (that is, Experiments 2-4 [neurological function, neurological cognition, and histopathology]).

Hematoma Consequences at 24 Hours (Experiment 1)

Animal Perfusion and Tissue Extraction—The animals were fatally anesthetized with isoflurane (≥ 5%) followed by cardiovascular perfusion with ice-cold PBS for the hemoglobin and Evans blue assays, and immunoblot analyses. The brainstem was then dissected and snapfrozen with liquid nitrogen, and then stored at −80°C, before spectrophotometric quantification or protein extraction. Because the hematoma was stereotactically induced, but edema is known to spread, the hemorrhage volume was quantified using only the extracted brainstem tissue, whereas brain water content was also measured in adjacent structures (that is, cerebral and cerebellar hemispheres).

Hemorrhagic Volume—The spectrophotometric hemoglobin assay was performed using well-established protocols.[14,119] Extracted brainstem tissue was placed in glass test tubes with 3 ml of PBS and then homogenized for 60 seconds (Tissue Miser Homogenizer, Fisher Scientific). Ultrasonication for 1 minute lysed erythrocyte membranes, the products were centrifuged for 30 minutes, and Drabkin reagent was added (Sigma-Aldrich) into aliquots of supernatant fluid that reacted for 15 minutes. Absorbance, using a spectrophotometer (540 nm; Genesis 10uv, Thermo Fisher Scientific), was calculated into a hemorrhagic volume on the basis of a standard curve.

Brain Water Content—The percentage of brain edema was measured using the wet-weight/dry-weight method.[119] Quickly after sacrifice, the brains were removed and divided. The tissue weights were determined before and after drying for 24 hours in a 100°C oven by using an analytical microbalance (model AE 100, Mettler Instrument Co.) capable of measuring with a 1.0-μg precision. The data were calculated as the percentage of water content: (wet weight − dry weight)/wet weight × 100.

Neuroscore—Composite neurological evaluation is a sensorimotor value consisting of the combined averages from wire suspension, beam balance, and inclined plane.[19,30] The Voetsch neuroscore (Table 2) is a modified vertebrobasilar scale score of sensorimotor ability.[47,123] The neuroscore was calculated as percentage difference (subtraction) of the mean performance from shams. Values are expressed as percentage of sham (further details provided in Experiment 2).

Physiological Variables and Vascular Permeability Measurement—Under general anesthesia (operatively), the right femoral artery was catheterized and physiological parameters were measured, reassessed 24 hours later on the opposite (left) side; this was followed by intravenous injection of 2% Evans blue (5 ml/kg, 1-hour circulation), as is routinely done.[64] Extracted brainstem tissue was then weighed, homogenized in 1 ml PBS, and finally centrifuged for 30 minutes. The supernatant fluid (0.6 ml) was added with equal volumes of trichloroacetic acid, and this was followed by a temporal pause and (biochemical) overnight incubation and recentrifugation the next day. The final supernatant

fluid underwent spectrophotometric quantification (615 nm; Genesis 10uv, Thermo Fisher Scientific) of extravasated dye, as previously described.[109]

Western Blot Analysis—For protein immunoblot,[119] the concentration of protein was determined using the DC Protein Assay (Bio-Rad). Samples were subjected to SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and then transferred to nitrocellulose membrane for 80 minutes at 70 V (Bio-Rad). Blotting membranes were incubated for 2 hours with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 and were then incubated overnight with the following primary antibodies: anticollagen IV (1:500; Chemicon), anti-ZO-1 (1:500; Invitrogen Corp.), anti-MMP-2 and anti-MMP-9 (1:1500; Millipore), followed by incubation with secondary antibodies (1:2000; Santa Cruz Biotechnology) and processing with the ECL Plus kit (Amersham Bioscience). Images were semiquantitatively analyzed using ImageJ (4.0, Media Cybernetics).

Neurofunctional Profiles at 30 Days (Experiment 2)

Animals were assessed using a battery of functional outcome measures. For the Voetsch neuroscore (maximum score 42), the sum of 14 parameters of a vertebrobasilar scale[47] was recorded (Table 2); scores for each parameter ranged from 3 (no neurological deficit) to 0 (complete neurological deficit). For locomotion, the path length in open-topped plastic boxes (49 cm long × 35.5 cm wide × 44.5 cm tall) was digitally recorded for 30 minutes and analyzed by Noldus EthoVision tracking software.[42,64] For sensorimotor function, the falling latency was recorded (60 second cut-off) when all four limbs were placed perpendicularly onto a stationary horizontal beam balance (50 cm in length × 5 cm in diameter) or as the forelimbs grasped onto the wire suspension (40 cm in length × 3 mm in diameter).[19] The inclined plane consisted of a box (70 cm long × 20 cm wide × 10 cm tall) with an analog protractor and hinged base, elevated at 5° intervals until the animal slipped backward.[30]

Neurocognitive Ability at the 3rd Week (Experiment 3)

Higher-order brain function was measured using a panel of specific tests. For the rotarod task, a motor-learning paradigm was assessed by comparing preoperative performance with four daily blocks on the 3rd week after injury on Days 21, 23, 25, and 27.[42,62] The apparatus consisted of a horizontal rotating cylinder (7 cm in diameter × 9.5 cm in width, acceleration 2 rpm/5 sec) requiring continuous walking to avoid falling, which was recorded by photobeam-circuit (Columbus Instruments). The T-maze assessed short-term (working) memory.[50] Rats were placed into the stem (40 × 10 cm) of a maze and allowed to explore until one arm (46 × 10 cm) was chosen. From the sequence of 10 trials, of left and right arm choices, the rate of spontaneous alternation was calculated (range 0% [no alteration/trial] to 100% [complete, alternations/trial]).[28,136] For the Morris water maze task, spatial learning and memory were assessed over 4 daily blocks on Days 22, 24, 26, and 28.[42,62] The apparatus consisted of a metal pool (diameter 110 cm), filled to within 15 cm of the upper edge, with a platform (diameter 11 cm) for the animal to escape onto that changed location for each block (maximum 60 sec/trial); trials were digitally analyzed using Noldus EthoVision tracking software. Cued trials measured place learning with the escape platform visible above water; spatial trials measured spatial learning with the platform submerged; and probe trials measured spatial memory once the platform was removed.

Histopathology at 30 Days (Experiment 4)

The animals were terminally anesthetized with isoflurane (≥ 5%) and transcardially perfused with ice-cold PBS and then 10% paraformaldehyde. The brains were removed and postfixed in 10% paraformaldehyde, followed by 30% sucrose (weight/volume) for 3 days. All

histopathological analyses used 10-μm-thick coronal sections caudally cut every 800 μm on a cryostat (LM3050S, Leica Microsystems) that were mounted and stained on poly-L-lysine-coated slides. Morphometric analysis of cresyl violet slides[64] involved computer-assisted (ImageJ 4.0) hand delineation of the ventricle system (lateral, third, cerebral aqueduct, and fourth), cerebellum (ipsi- and contralateral), brainstem (midbrain, pontine, and medulla), and lesioned area (cavity, cellular debris). Borderlines were based on criteria defined from stereological studies that used optical dissector principles.[2,5,27,94,103,120] Brain tissue volumes were calculated over 8 sequential sections (every 800 μm; 7.4-13 mm from the bregma) using established methods: [(average [(area of coronal section) − (lesion area)] × interval × number of sections)].[71] To account for variant neuronal densities in the brainstem (midbrain, pontine, and medulla), the relative loss of cell density was estimated using established protocols,[71,99,111,113] where the cells were counted using ×400 magnification over 5 equidistant areas (250 × 250-μm grids) per brainstem side (ipsi- and contralateral).[29,64] The relative loss of neurons was then determined by subtracting the difference in averages of the two sides,[62] and the final value was expressed as the neuronal losses per brainstem region. All measurements were performed blinded and twice repeated.

Statistical Analysis

Statistical significance was considered at an α-level of $p < 0.05$. Data were analyzed using ANOVA, and with repeated-measures ANOVA for the long-term neurobehavior analysis. Significant interactions were then further explored with the conservative Scheffe post hoc test, t-test (unpaired), and Mann-Whitney rank sum test, when appropriate.

Results

Experimental Model of Pontine Hemorrhage in the Rat

Systemic physiological parameters were stable during and after (1 and 30 days) the surgical procedure (Table 1). Under isoflurane anesthesia, the nonparalyzed animals breathed spontaneously (no ventilator), and blood gases were monitored intra- and postoperatively, as is routinely done during experimental rat collagenase brain infusion studies.[42,64] Within 30 minutes of waking from anesthesia, the collagenase pontine-infused animals, as determined by our qualitative observations, exhibited ataxic gaits, craniocaudal rotations, and cranial nerve and limb dysfunctions. These neurological deficits, similar to those previously described following posterior brain circulation injury in rats,[47] formed the basis of our qualitative neuroscore, as defined in Table 2. Repeated gross pathological examination (during tissue preparation for biochemical or morphological analyses) revealed well-localized hematomas within the right ventral tegmental pons, without subarachnoid (or subdural) bleeding and without any ventricular obstruction (Fig. 1B and C). The timing of animal deaths in this study is demonstrated using the Gehan-Breslow survival graphs (Fig. 1D and E). The mortality rates were 8% and 25% at 24 hours (0.15 and 0.3 U, respectively [Fig. 1D]; Experiment 1), and 54% over 30 days (0.15 U [Fig. 1E]; Experiments 2-4). Non-survivor data were excluded from study.

Hematoma Consequences at 24 Hours (Experiment 1)

Unilateral collagenase infusion led to dose-dependent elevations of hematoma volume, brain water content, neurological deficits, and vascular permeability ($p < 0.05$; Fig. 2A-D). Immunoblots showed significant elevation of MMP-2 and -9 and degradation of collagen Type IV and ZO-1 ($p < 0.05$; Fig. 2E).

Neurofunctional Profiles Over 30 Days (Experiment 2)

Infusion of collagenase (0.15 U) led to significant neurological (modified Voetsch score), locomotor (open field), and sensorimotor (wire suspension, beam balance, and inclined plane) deficits over the 1st week postinjury ($p < 0.05$; Fig. 3A-F), in spite of stable body weights ($p > 0.05$). Most functional parameters recovered before the 3rd week ($p > 0.05$) after collagenase infusion, except for that measured for the inclined plane, which continued to show differences compared with the controls ($p < 0.05$; Fig. 3F).

Neurocognitive Ability at Week 3 (Experiment 3)

Collagenase (0.15 U)-infused animals performed significantly worse than controls over all postoperative rotarod (motor) testing blocks ($p < 0.05$; Fig. 4A) and were unable to improve upon their preoperative performance ($p > 0.05$), whereas the controls progressively improved their falling-latency time ($p < 0.05$). Although T-Maze results showed a significant loss of “working” memory ($p < 0.05$; Fig. 4B), the water-maze demonstrated equal “place learning” (cued trials) for all groups ($p > 0.05$; Fig. 4C). On the spatial blocks, however, collagenase-infused animals performed significantly worse than controls ($p < 0.05$) and were unable to improve upon their place-learning performance ($p > 0.05$). In comparison, controls performed progressively better with each block ($p < 0.05$; Fig. 4C) and with the spatial memory probe ($p < 0.05$; Fig. 4D).

Histopathology at 30 Days (Experiment 4)

The topography of the right tegmental pontine lesion extended within the brainstem in a Gaussian distribution, surrounding the level of injection (10.2 mm caudal from the bregma; Fig. 5A). The cystic cavitary lesion caused focal atrophic losses of brainstem tissue volume ($p < 0.05$; Fig. 5B, C, and E), without affecting the size of the cerebellar hemispheres ($p > 0.05$; Fig. 5C). The ventricles remained patent and unchanged in size ($p > 0.05$; Fig. 5D). Quantifiably, the greatest percentage of atrophy was pontine (36.9% ± 6.2%, $p < 0.05$; Fig. 5E), in comparison with the midbrain (16% ± 5.3%) and medulla (14% ± 5.7%). However, the neuronal densities were diffusely diminished (pontine 8.63 ± 2.7; midbrain 5.35 ± 2.8, medulla 5 ± 2.2; Fig. 5F).

Discussion

A requisite priority of translational stroke research is the characterization of appropriate experimental ICH models.[87] Animal models of posterior circulation hemorrhage help demonstrate the unique features of cerebellar[64] and pontine[66] lesions in comparison with other brain injury sites.[67] This is particularly important because clinical studies do not adequately address this patient population.[72,73] The use of autologous blood or collagenase infusion into the basal ganglia region of rodents represents the currently most common experimental approaches for studying this condition.[1,71,129] However, basal ganglia hemorrhage is a deep cerebral bleeding subtype that, as a group (basal ganglia, thalamus, and internal capsule), comprises no more than half of all ICH cases.[32] Basic research translation to clinical studies has given little attention to posterior stroke.[17,18,34,41,80] The duality of being too uncommon to achieve sufficient numbers in some patient centers, confounded by clinicopathological heterogeneity[39] between posterior and anterior circulation stroke, makes experimental models representing this condition all the more needed. Animal models representing the full spectrum of ICH are thus needed.[70,116]

Over the past half century, surgical approaches for brainstem hemorrhages have been widely debated in the literature.[6,46,54,55,57,58,61,83,86,89,90,96,118] In fact, the report of successful pontine infusion into the rat pons tegmentum was what first prompted the development of this model.[91] However, this infratentorial (pontine hemorrhage) patient population has been

excluded from what will arguably be one of the greatest pivotal clinical studies on surgical treatment of ICH in a decade.[79,80] Furthermore, this study is an extension of our infratentorial hemorrhage rodent studies of the cerebellum[64,65] and builds on our preliminary report describing the feasibility of experimental pontine hemorrhage.[66] Early outcomes after pontine hemorrhage are dependent on the extent of the initial bleed[85,127] more than anything else.[9] The use of collagenase thus relates well to the study of the human clinical PPH condition,[78] because many have reproducibly demonstrated the utility of collagenase in the evaluation of strategies to mitigate early hematoma expansion.[104] The clinical relevance of this animal model relates to the fact that pontine hemorrhage represents such a small percentage of ICH—thus making high-quality clinical studies difficult to perform and highlighting the need of an instructive animal model to evaluate strategies to prevent and treat posthemorrhagic complications.

The early hematoma consequences in the present study were in agreement with experimental findings of basal ganglia ICH in rats with regard to collagenase-induced edema, neurodeficit, blood-brain barrier rupture, and elevation of MMP-2 and -9 levels.[100,106,126] Our immunoblots demonstrated relative changes in the protein expression of MMP subtypes, while further study using gel zymography will define the change in MMP-2 and -9 enzymatic activity. In further limitation, future work will need to determine any temporally related changes to cortical cerebral blood flow, and intracranial pressures, just as these changes were determined following related experimental subarachnoid hemorrhage in rats.[51] Nonetheless, this model demonstrated consistent neurobehavioral outcomes using 0.15-0.3 U of collagenase, much like the basal ganglia ICH using 0.2 U of collagenase in rats.[71] This is well below the amount known to cause significant neurotoxicity.[76] Thus, both doses of collagenase produced adequate neurological injury, and we chose the lower dose for long-term evaluations. This minimized any additional collagenase contribution to inflammation[125] and also prevented any component of vestibular syndrome (head tilt or rolling) at the 30-day evaluation. The robust performance of lesioned animals in finding the platform in the cued water maze trials confirmed the lack of any significant visual-vestibular disturbance. On the other hand, the infusion of collagenase is likely to ultimately affect both the composition of the extracellular matrix and the migration of inflammatory cells. This may therefore limit the use of this model to explore treatments aimed at modulating the inflammatory response after pontine hemorrhage. Future studies may therefore evaluate additional molecular mechanisms as interventional strategies for the early hematoma, neurobehavioral deficits, and neurovascular remodeling.[106,132,135]

Importantly, the collagenase infusion yielded long-term neurobehavioral patterns similar to clinical reports after survivable pontine hemorrhage in humans.[22,127] Almost one-half of survivors from pontine hemorrhage will retain long-term deficits across motor-learning and visuospatial neurocognitive domains, even after full rehabilitation from paralytic losses of overall strength, muscle tone, and coordination.[74,75,88] In agreement, our study demonstrated that most neurological impairments resolved over the first 3 weeks of assessments, while motor-learning (rotarod) and cognitive ability (water maze and T-maze) remained impaired up to 1 month later. Similar neurobehavioral outcomes were confirmed, having been described in other experimental brainstem lesion models using rats.[23,36,40,115,122] Interestingly, the finding of marginal (inclined-plane) hindlimb recovery diverged from both clinical and previously known experimental manifestations. This is possibly a rodent (and model)-specific feature, since the lateral placement of our 30-day ventral pontine tegmentum lesion anatomically obliterates the rodent hindlimb control region, while the forelimb neuronal tracts are located more medially in the rodent brainstem.[81,107] Also, the lateral pons-brainstem contains cerebropontocerebellar projections that link hindlimb control regions of the cerebral cortex and cerebellum together to form the largest sensorimotor structure within the mammalian brain.[92] Therefore any obliteration

along these neural tracts could lead to lasting hindlimb deficits, similar to those found after cerebellar hemorrhage in rats.[64]

As previously mentioned, there have been several prior reports about cognitive deficits following infratentorial lesions, and the topic of clinically translating such data has been extensively reviewed.[42,63,64,67,104] Notwithstanding, clinical cognitive dysfunction following brainstem hemorrhage in humans remains a weakly reported fact in the literature,[74,88] and our study further highlights the novelty and importance of using such a rodent ICH model to examine an important clinical outcome of which little is known.[22] However, in terms of specifically why cognitive deficits were observed following pontine hemorrhage, one can only speculate. Although we were not surprised to find that pontine lesions were associated with sensorimotor, coordination, and motor-learning deficits (as seen in the rotarod), we acknowledge that finding cognitive deficits following a pontine lesion was unexpected. Nevertheless, the animals generally appeared normal while walking or swimming, and we are confident that the deficits described were due to cognitive, rather than sensorimotor and/or coordination, issues. First, the cued task provides a built-in behavioral control for the water maze test.[43] The observation that all groups performed equally when the escape platform was visible suggests that all groups were equally motivated to find the platform, that they could see the platform, and that they could easily swim to the platform. Only when the platform was submerged (requiring the use relational/contextual learning strategies) did the behavior of the pons group decline. This pattern of activity (unimpaired cued behavior with a visible platform, but impaired performance with a hidden platform) represents the classic spatial-learning deficit.[84] Second, also during the probe tasks in which the platform was removed, only the pontine group swam randomly around the tank, whereas only the control groups exhibited targeted searches in the quadrant that previously contained the platform. Again, this pattern of behavior is representative of the classic spatial memory deficit.[44] Third, during these probe trials, all groups swam at a similar speed and covered a similar distance, signifying the lack of neurological-functional differences between the groups for this specific task. The finding of impaired working memory performance on the T-maze task further supports this interpretation, because walking down the T-maze was easy enough for all rats and yet only the pontine group exhibited random performance, which is classically interpreted, in the absence of any overt motor issues that would inhibit performance, as an impaired working memory.[28]

The neuropathology after pontine hemorrhage has been poorly described, but it is necessary for the establishment of the therapeutic window and for the choice of drug. Therefore, we related our neurological outcomes with the histopathological substrates, as a morphological measure of the brain injury.[29,48] Thirty days after collagenase infusion, we found a well-circumscribed atrophic cavitary lesion in the right pons-brainstem. The atrophy was focal (nonspreading), not significantly affecting the rostral (midbrain), caudal (medulla), or dorsal (cerebellar hemisphere) structures. Such focal cystic cavitary lesions similarly occur after experimental basal ganglia ICH in rats.[29,71] However, we did find diffuse (nonfocal) losses of neuronal density in the midbrain, pontine, and medulla 1 month after collagenase infusion (0.15 U). These neuronal tissue losses likely contributed to the causation of delayed neurocognitive and hindlimb deficits. Further studies will need to investigate the involvement of specific nuclei or fascicles, to describe the key structures involved in the pons, and the related anatomical areas in the brainstem and in the cerebellum—to specifically describe which structures were destroyed, which nucleus lost its neurons, and which fascicles degenerated. There is a need to evaluate the role of neuronal apoptosis, the possible timelines of Wallerian degeneration, and the pattern of inflammation and astrogliosis. Taken together, the combined networks of linked neuropathological injuries after pontine hemorrhage represent potential treatable lesions using therapeutic neuroprotective strategies.[110]

To date, all the preclinical ICH studies to improve functional outcome have been performed using models of basal ganglia hemorrhage, and these interventions have been extensively reviewed in a recent meta-analysis.[35] An impressively scant amount is known about the brainstem with regard to potentials in neuroprotection, neuroplasticity, and the utility of stem cells or pharmacological approches.[25,72,73] To highlight the differences, compared with the more common basal ganglia hemorrhages (ICH) having less than 50% mortality rates,[7] PPH is highly lethal, leading to a mortality rate in excess of 65%.[15,31,137] This much greater severity is likely related to the many ascending and descending brainstem projections connected with the reticular formation, cardiovascular, and respiratory centers (reviewed by Lekic et al.[63]). Current therapeutic approaches following PPH, however, are largely based on outcomes found in basal ganglia hemorrhage.[72,73] Comparatively, whereas up to 80% of rehabilitating ICH patients are not yet living independently by 6 months following stoke ictus,[37] deficits in most PPH events are so severe that the majority of patients may not even qualify to enter rehabilitation because of such a poor prognosis and survival rate.[108] As the basis for this and future research, this PPH animal model can relevantly address the goals of clinically improving the rates of mortality, overall morbidity, and ultimate functional recovery using future experimental studies.

Brainstem nuclei are anatomically scattered in a mosaic of physiologically relevant neuronal islands.[2,5,94,103,120] A gerbil model of hindbrain ischemia has shown the greatest numbers of cell deaths near areas of coordination and balance (cerebellar interpositus and lateral vestibular nuclei) while cardiorespiratory foci had seemingly greater “innate” neuroprotection.[45] Similarly, our study found lasting neurobehavioral deficits, while (cardiorespiratory) physiological variables remained intact up to 30 days later. Loss of these life-sustaining nuclei may, however, explain our 30-day mortality rate (approximately 50%), which is analogous to the clinical findings.[137] Clinically, brainstem neurons located within the area postrema contribute important regulations in terms of cardiovascular functions.[53,101,112,134] This brain region expresses cell-surface receptors for circulating molecules (angiotensin II and vasopressin), and likely has some control over cerebrovascular activity.[38,49,68] In the brainstem, the angiotensin II hormone resets the baroreflex to a higher blood pressure level through indirect interactions with the solitary tract nucleus and through interconnections to the medulla.[77,95,131] These nuclei also modulate the cardiovascular regulation of other neuropeptides, such as vasopressin; this homeostatic effector can readily bind to somatic V2-type receptors, causing peripheral vasoconstriction. On the other hand, vasopressin receptor binding interactions at the area postrema will paradoxically enhance the baroreflex set-point to an activation level of lower threshold pressure.[20,59,93] Experimental studies further reveal significant cerebellar fastigial nuclei involvement in this cerebrovascular control.[69,82,130] The interaction occurs through an integration of autonomic signals arising from adjacent brainstem vestibular and Purkinje neurons.[24,133] As part of a larger brainstem network, the cerebellar fastigial nuclei modulate function of medullary structures and autonomic spinal intermediolateral column neurons.[11,21] In primates, these nuclei circuits are pathways to interconnect the vestibular (lateral and inferior), reticular (lateral, paramedian, and gigantocellular), and cervical spinal anterior gray neurons.[4] Depending on the type of infratentorial brain injury, experimental studies have demonstrated that electrical stimulation of the fastigial nuclei leads to pressor responses with tachycardia (mediated by fibers passing through, or very close to, the nuclei), while chemical activation causes bradycardia depressor responses through intrinsic fastigial nuclei neuronal activity.[12,13,97] Taken together, these clinically relevant human infratentorial circuitry pathways help keep the balance of complex cerebrovascular systems and underscore the importance of better understanding localized lesions to this brain region. This experimental PPH model thus represents a means of impacting future patient care through the study of specific surgical, pharmacological, and regenerative approaches.

Conclusions

We have characterized the early brain injury, neurobehavioral profiles, and histopathology in a highly reliable and easily reproducible experimental model of pontine hemorrhage using rats. Therapeutic strategies that mitigate the early mass effect (hematoma growth and brain edema), or promote lasting neuroprotection (neurobehavioral and atrophic remodeling) could lead to clinical approaches that afford these patients better outcomes in the future.[102,124,129] While the present study has well characterized the neurological deficits, particularly during the chronic phase, pathophysiological investigations are needed to help establish the therapeutic window for mitigating early (and delayed) damage, the choice of drugs, and the appropriate design of stroke rehabilitation studies. These findings provide a basis for the establishment of these pathophysiological features, serving as a foundation on which to perform further therapeutic investigation.

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Abbreviations used in this paper

ICH	intracerebral hemorrhage
PBS	phosphate-buffered saline
PPH	primary pontine hemorrhage

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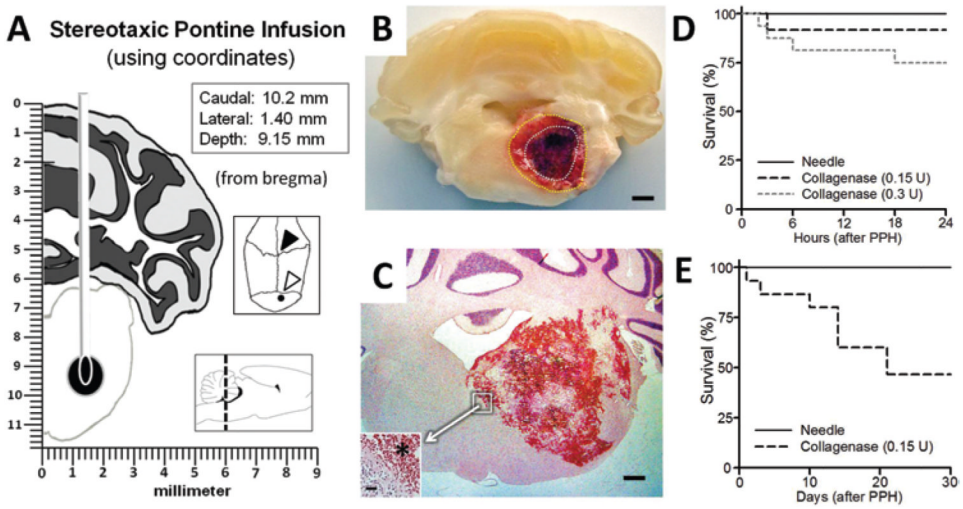

Fig. 1.

Experimental model of pontine hemorrhage in the rat. **A:** Schematic showing stereotactic needle placement into the right ventral pontine tegmentum (**Insets:** coordinates [upper]; cranial aspect of bregma *[open arrowhead]* and the infusion site *[closed arrowhead]*; sagittal demonstration with the *dotted line* at craniocaudal level of infusion [lower]). **B:** Photograph of coronal section of tissue at 24 hours after collagenase (0.15 U) infusion, showing the hematoma surrounded by vasogenic edema (outlined with *dotted lines*). Bar = 1 mm. **C:** Representative H & E-stained cryosection (12 hours, 0.3 U collagenase) illustrating the hemorrhage. Bar = 0.5 mm. (**Inset** showing the hematoma border *[asterisk]*. Bar = 20 μm.) **D and E:** Gehan-Breslow survival analysis results at 24 hours and 30 days.

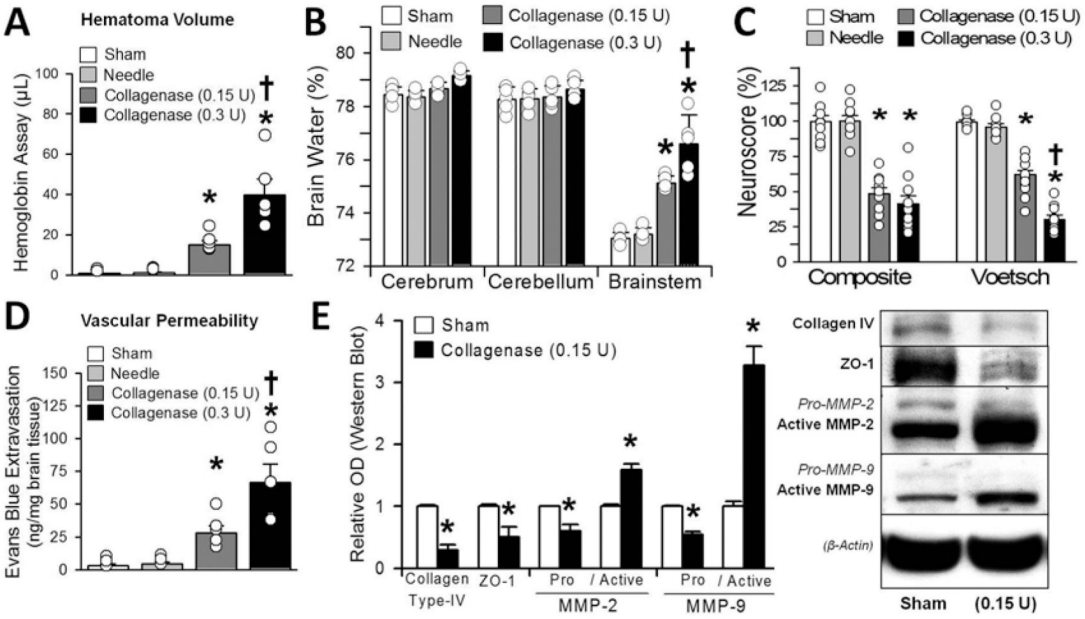

Fig. 2.

Hematoma consequences at 24 hours: hematoma volume assay **(A)**, brain water **(B)**, neuroscore (composite and Voetsch) **(C)**, vascular permeability **(D)**, and **(E)** semiquantification (left) of the collagen Type IV, ZO-1, and MMP-2 and -9 protein immunoblots (right). Values are expressed as the mean ± SEM (n = 10 [neuroscore] and n = 5 [all others]). *$p < 0.05$ compared with controls (sham and needle trauma). †$p < 0.05$ compared with collagenase infusion (0.15 U). *Circles* in *bars* indicate the raw data points of each measurement, and *whiskers* represent the SEM. OD = optical density.

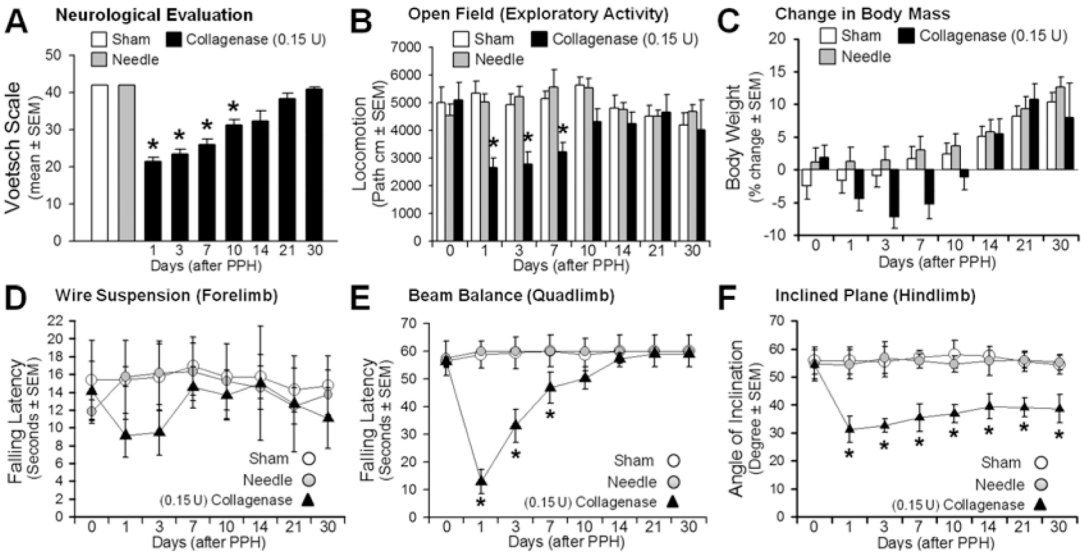

Fig. 3.

Neurofunctional profiles in 30-day period: Voetsch score **(A)**, locomotion **(B)**, body weight **(C)**, wire suspension **(D)**, beam balance **(E)**, and inclined plane **(F)**. Values are expressed as mean ± SEM (n = 8 [sham and needle trauma] and n = 7 [collagenase infusion, 0.15 units]). *$p < 0.05$ comparing collagenase infusion *(closed triangles)* with controls (sham, or needle trauma, *open circles*). path = path length.

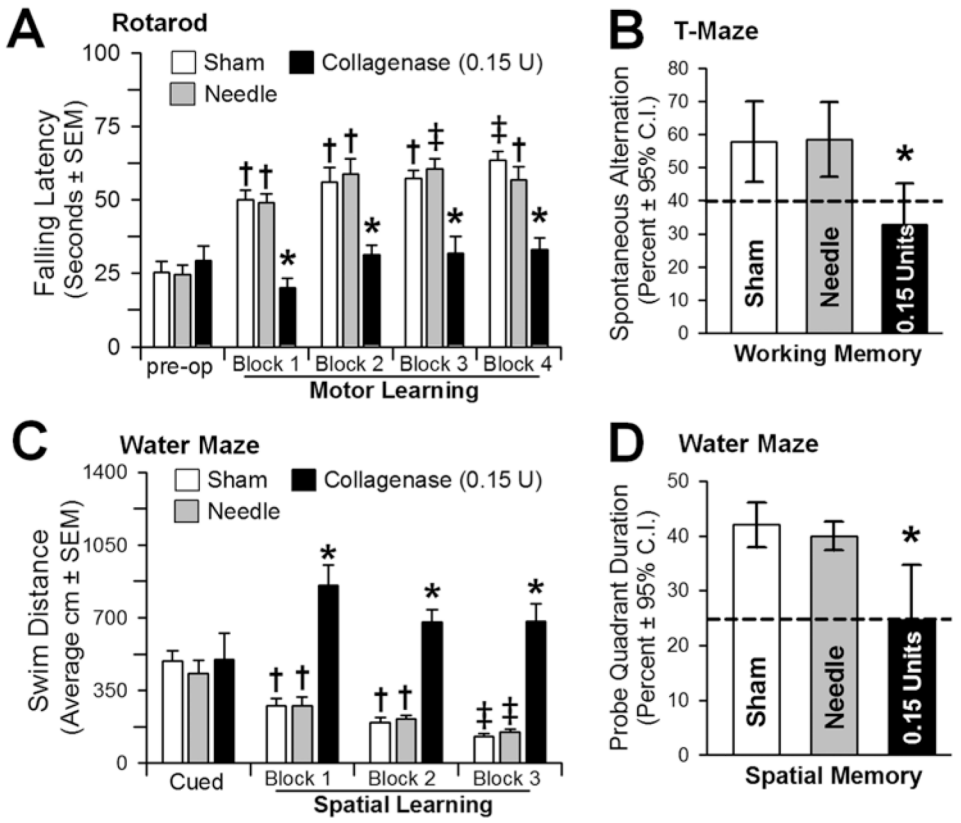

Fig. 4.

Neurocognitive ability at 3 weeks. **A:** Motor learning assessed by the change in the latency to falling off an accelerating rotarod (2 rpm/5 sec) preoperatively and across 4 daily blocks. **B:** Working memory quantified by the number of spontaneous alternations in the T-maze. **C:** Spatial learning assessed by the swim distance needed to find the visible (cued) versus the hidden (spatial) platforms in the water maze. **D:** Spatial memory was determined by the percent duration in the probe quadrant when the platform was removed. Values expressed as the mean ± SEM (rotarod, cued and spatial water maze) and mean ± 95% CI (T-maze and probe quadrant), n = 8 (sham and needle trauma), n = 7 (collagenase infusion, 0.15 U). *$p < 0.05$ compared with controls (sham and needle trauma). †$p < 0.05$ compared with preoperative (rotarod) or cued trials (spatial water maze). ‡ $p < 0.05$ compared with Block 1 (rotarod and spatial water maze).

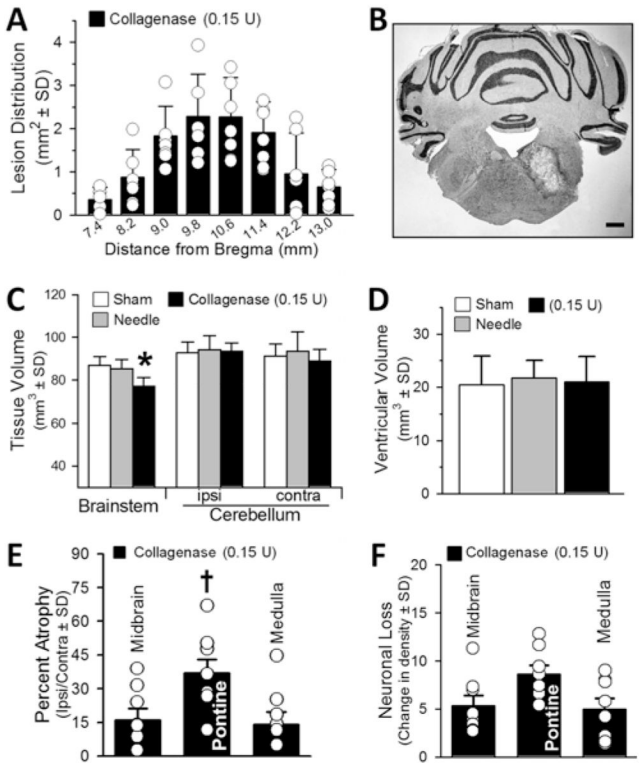

Fig. 5.

Histopathological results at 30 days. **A:** Lesion distribution (in mm^2) throughout the brainstem. **B:** Representative cryosection illustrating the cystic cavitary lesion. Bar = 1 mm. **C:** Infratentorial brain tissue volume (in mm^3). **D:** Volume of the ventricles (in mm^3). **E:** Percentage of atrophy of the brainstem (midbrain, pontine, and medulla), expressed as the volumetric difference between ipsilateral and contralateral sides. **F:** Neuronal density loss over the intact (perilesional) brainstem regions expressed as average difference in cell counts between ipsilateral and contralateral sides. Values expressed as the mean ± SD (n = 8 [sham and needle trauma] and n = 7 [collagenase infusion, 0.15 U]). *$p < 0.05$ compared with controls (sham and needle). †$p < 0.05$ compared with midbrain and medulla (percentage of atrophy).

TABLE 1

Summary of physiological variables during and after collagenase infusion*

	Arterial Blood Gas				
Group	**pH**	**PO_2 (mm Hg)**	**PCO_2 (mm Hg)**	**BP (mm Hg)**	**HR (per min)**
during Tx					
sham	7.33 ± 0.03	186 ± 15	45 ± 3	81 ± 6	348 ± 40
needle	7.39 ± 0.02	184 ± 21	43 ± 5	89 ± 6	369 ± 28
CI—15 U	7.34 ± 0.05	192 ± 11	44 ± 8	89 ± 8	355 ± 33
CI—0.3 U	7.32 ± 0.03	187 ± 5	46 ± 5	91 ± 5	362 ± 25
30 mins after Tx					
sham	7.32 ± 0.01	185 ± 20	48 ± 5	84 ± 6	347 ± 48
needle	7.38 ± 0.05	186 ± 14	44 ± 3	88 ± 8	359 ± 14
CI—0.15 U	7.30 ± 0.08	188 ± 10	48 ± 9	88 ± 10	351 ± 33
CI—0.3 U	7.29 ± 0.03	173 ± 17	50 ± 9	90 ± 5	380 ± 37
24 hrs after Tx					
sham	7.50 ± 0.04	192 ± 12	36 ± 5	84 ± 5	348 ± 20
needle	7.52 ± 0.02	181 ± 7	37 ± 7	78 ± 3	361 ± 17
CI—0.15 U	7.47 ± 0.03	188 ± 11	38 ± 4	85 ± 9	359 ± 34
CI—0.3 U	7.38 ± 0.06	179 ± 14	49 ± 9	90 ± 9	329 ± 46
30 days after Tx					
sham	7.5 ± 0.06	182 ± 28	37 ± 8	94 ± 4	369 ± 9
needle	7.47 ± 0.03	192 ± 12	36 ± 4	83 ± 5	364 ± 27
CI—0.15 U	7.47 ± 0.03	190 ± 44	35 ± 4	87 ± 2	358 ± 39

*There was no statistically significant difference between groups. Values are presented as the mean ± SD. Abbreviations: BP = blood pressure; CI = collagen infusion; HR = heart rate; Tx = treatment.

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TABLE 2
Summary of the modified Voetsch neuroscores

	Score			
Test	**3**	**2**	**1**	**0**
head movement (spontaneous)	moves in all dimensions	prefers 1 side	only to 1 side	flexed to 1 side
lethargy (confrontation)	fully responsive	moderately responsive	minimally responsive	coma
hearing (auditory startle)	finger rub	snap of fingers	loud clap	no startle
pain reflex (ear pinch)	brisk & symmetrical reaction	slightly diminished or asymmetri-cal reaction	greatly diminished & asymmetri-cal reaction	no reaction
corneal reflex (sensorimotor)				
proprioception (vibrissae)				
sensation (neck)				
exploration (5 mins in cage)	reaches 2 walls	reaches 1 wall	moves along base	no movement
circling (craniocaudal)	bilat turns	prefers 1 side	only to 1 side	falls to 1 side
sensation (axial)	brisk & symmetrical reaction	slightly diminished or asymmetri-cal reaction	greatly diminished & asymmetri-cal	no reaction
4-limb movement (outstretch)	equal bilat	slight asymmetry	great asymmetry	paresis
forelimb movement (outstretch)				
climbing (motor function)	climbs to top	impaired climbing	stationary grip	falls immediately
beam (motor function)	explores both ends	some movement		