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Research paper

# rhIGF-1 reduces the permeability of the blood-brain barrier following intracerebral hemorrhage in mice

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ABSTRACT

Disruption of the blood-brain barrier results in the formation of edema and contributes to the loss of neurological function following intracerebral hemorrhage (ICH). This study examined insulin-like growth factor-1 (IGF-1) as a treatment and its mechanism of action for protecting the blood-brain barrier after ICH in mice. 171 Male CD-1 mice were subjected to ICH *via* collagenase or autologous blood. A dose study for recombinant human IGF-1 (rhIGF-1) was performed. Brain water content and behavioral deficits were evaluated at 24 and 72 h after the surgery, and Evans blue extravasation and hemoglobin assay were conducted at 24 h. Western blotting was performed for the mechanism study and interventions were used targeting the IGF-1R/GSK3β/MEKK1 pathway. rhIGF-1 reduced edema and blood-brain barrier permeability, and improved neurobehavior outcomes. Western blots showed that rhIGF-1 reduced p-GSK3β and MEKK1 expression, thereby increasing occludin and claudin-5 expression. Inhibition and knockdown of IGF-1R reversed the therapeutic benefits of rhIGF-1. The findings within suggest that stimulation of the IGF-1R is a therapeutic target for ICH which may lead to improved neurofunctional and blood-brain barrier protection.

## 1. Introduction

Intracerebral hemorrhage (ICH) is one of the most dangerous forms of stroke that currently has no effective treatments. Among the several pathological sequelae of ICH, the hematoma-induced breakdown of the blood-brain barrier (BBB) is one of the major concerns which plays a critical role in the pathophysiology of the disease (Selim and Sheth, 2015; Xi et al., 2002). Loss of endothelial tight junction proteins contribute to increased permeability of the BBB leading to the formation of vasogenic edema and a deterioration of neurological functions (Liu et al., 2012; Gebel Jr et al., 2002). Previous studies have demonstrated that the protection and recovery of tight junction proteins decreases the severity of the ICH injury with improved neurological outcomes (Krafft et al., 2013).

Insulin-like growth factor 1 (IGF-1) is highly selective for the IGF-1 receptor (IGF-1R), which is expressed on several cell types in the brain including neurons, astrocytes, and endothelial cells (Bake et al., 2016; Liu et al., 1994; Shemer et al., 1987). Canonically, stimulation of the IGF-1R causes of the phosphatidylinositol 3-kinase (PI3K)-Akt pathway thus attenuating glycogen synthase kinase-3β (GSK3β) activation (Hetman et al., 2000; Allen et al., 2005). Previously, our lab demonstrated that preventing activation of glycogen synthase kinase-3β (GSK3β) reduces edema and damage to the BBB following injury through protecting tight junction proteins (Krafft et al., 2013). GSK3β physically binds to and activates mitogen-activated protein kinase (MAPK) kinase (MEK) kinase 1 (MEKK1), an important stimulator of the c-Jun/JNK pathway, which has been shown to increase MMP9 production (Kim et al., 2003; Feiler et al., 2011). GSK3β has also been shown to reduce expression of the tight junction proteins occludin and claudin-5, indicating an increase in the permeability of the barrier (Nitta et al., 2003).

IGF-1 has been previously described to have protective effects in ischemic brain injury models, however the role of this growth factor remains unexplored in ICH. In addition, the role and mechanism by which IGF-1 and IGF-1R protects the BBB is not completely understood. We hypothesize that IGF-1 will decrease the permeability of the BBB and reduce edema in an ICH mouse model *via* IGF-1R stimulation, reducing GSK3β/MEKK1 activation, to prevent the loss of tight junction protein expression.

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## 2. Materials and methods

### 2.1. Animal care

All procedures were done in accordance to institutionally approved protocols and the NIH Guide for Critical Care and Use of Laboratory Animals. Male CD-1 mice from Charles River, Wilmington, WA were housed in a light and temperature controlled room with access to water and food.

### 2.2. Intracerebral hemorrhage surgery

Intracerebral hemorrhage was induced either by sterile collagenase type VII infusion or a double injection of autologous blood both which we have previously utilized (Krafft et al., 2013; Rolland et al., 2013). Mice were anesthetized using an intraperitoneal (i.p.) co-injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). An incision on the scalp was made to reveal the location of bregma and the mouse then positioned on a stereotactic head frame (Stoelting Company, Wood Dale, IL). A small burr hole was created (coordinates from bregma: 0.2 mm anterior, 2.2 mm right lateral) for the insertion of a 27-gauge Hamilton needle (Hamilton Company, Reno, NV). A 10 μl or 250 μl Hamilton needle was filled with collagenase or autologous whole blood respectively and lowered into the burr hole. For the collagenase injection model, 0.075 U (dissolved in 0.5 μl of PBS) of collagenase was infused into the right hemispheric basal ganglia at a rate of 0.1667 μl/min and a depth of 3.5 mm. For the autologous blood injection model, 5 μl of blood was first injected at a rate of 2 μl/min and depth of 3.0 mm. The needle was then lowered to 3.7 mm where following a 5-minute wait, 25 μl was injected at a rate of 2 μl/min. All infusions were controlled with a micro perfusion pump (Harvard Apparatus, Holliston, MA). Following the infusions, the needle was left in place for 5 min before being retracted at a rate of 1 mm/min. Sham operated animals were only subjected to the needle insertion. Following the retraction of the needle, the burr hole was sealed using bone wax and the scalp sutured. All animals received subcutaneous (s.c.) injections of 0.2 ml of saline into each flank (0.4 ml total) while the mice were placed on a warm heating pad for recovery and observation. During the surgery, all animals were temperature regulated using a heating pad with a rectal probe.

### 2.3. siRNA intracerebroventricular injection

Two different IGF-1R siRNAs (Cell Signaling and Santa Cruz Biotechnology) were mixed and administered *via* intracerebroventricular (i.c.v.) injection (Ma et al., 2011). Briefly, both siRNAs were prepared following the manufacturer's instructions by dissolving in sterile RNase-free water. Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) i.p. co-injection. The IGF-1R siRNA mixture and scramble siRNA (100 pmol) was administered *via* i.c.v. 24 h before ICH induction as previously described (Yu et al., 2017; Zhao et al., 2018). A small burr hole was created at 1.0 mm left lateral from bregma and a 27-gauge Hamilton needle lowered to 2.3 mm deep and infused at 0.67 μl/min. The needle was left to rest for 5 min and then retracted at a rate of 1 mm/min. Following the retraction, the burr hole was sealed with bone wax and scalp sutured.

### 2.4. Experimental groups and pharmacological interventions

A total of N = 171 (Experimental N = 168; Exclusions N = 3) animals were used in this study with 3 animals excluded due to mortality during the surgery procedure (1 Vehicle, 1 rhIGF-1 + AG1024, 1 rhIGF-1 + scramble siRNA). Experimental groups consisted of mice subjected to either sham surgery (N = 30), collagenase-induced ICH (n = 126), or autologous whole blood-induced ICH (N = 12). Animals were randomly selected for treatments following the ICH surgery. Recombinant human IGF-1 (rhIGF-1; Cell Signaling) was prepared in concentrations of 10 μg (N = 12) or 50 μg (N = 66) in 12 μl of PBS and given through intranasal (i.n.) administration 3 h after the surgery (Fletcher et al., 2009; Cai et al., 2011). Alternatively, a daily dose of 50 μg rhIGF-1 (N = 6) was given starting 3 h after the surgery up to 72 h after the surgery. AG1024 (Santa Cruz Biotechnology), a specific inhibitor of the IGF-1R, was given in 40 μg/100 μl through i.p. injections 1 h after the surgery (Chang et al., 2010; 2011). Preparation of scramble siRNA and IGF-1R siRNA was previously discussed. 50 μg rhIGF-1 was given alone or in combination with AG1024 (N = 12), scramble siRNA (N = 12), or mixed IGF-1R siRNA (N = 12). Temperature and heart rate were monitored for variations throughout the surgery as well as before neurological testing and animal sacrifice.

### 2.5. Neurobehavioral assessment

A blinded investigator performed all neurological testing. The modified Garcia Neuroscore, corner turn evaluation, and forelimb placement exam were utilized to examine sensorimotor deficits in mice at 24 and 72 h after the surgery (Hua et al., 2002). The modified Garcia Neuroscore evaluates the animals sensorimotor abilities using 7 tests including 1) spontaneous activity, 2) side stroking, 3) vibrissae proprioception, 4) limb symmetry, 5) lateral turning, 6) forelimb walking, and 7) climbing (Garcia et al., 1995). Performance for each individual test was graded on a scale of 0 (worst performance) to 3 (best performance) with the sum of the scores (max = 21) providing the overall performance of the animal. For the corner turn exam, animals advanced into a 30° corner and the direction rotated to exit the corner was recorded (10 or 20 total trials). A turn was counted when the animal completes its rotation by lifting its forepaws to the sidewall and then returning to the ground in a direction opposite of the corner. The overall performance was evaluated as the number of left turns/total turns × 100 (%). The forelimb placement exam evaluates the reflex motor capabilities of the animal. The animal is lightly restrained, and vibrissae-stimulation provided to both ipsilateral (10 trials) and contralateral sides (10 trials). The overall performance was recorded as the number of times the forelimb elicited a reaching response/number of trials × 100 (%). Mice that were sacrificed at 72 h were tested for neurobehavior at both 24 and 72 h following the surgery.

### 2.6. Measuring brain water content

Brain water content was evaluated at 24 and 72 h after the ICH surgery using the wet/dry method (Ma et al., 2011). Briefly, mice were anesthetized with isofluorane and decapitated. The brains were quickly removed, and a coronal brain section of 4 mm centered on the needle tract was separated into ipsilateral and contralateral cortex and basal ganglia regions as well as the cerebellum for individual analysis. All sections were weighed on an analytical microbalance immediately to produce a wet weight and after 24 h (100 °C) to get a dry weight. To calculate the brain water content, the formula used was: (wet weight-dry weight)/wet weight × 100.

### 2.7. Evans blue extravasation

Blood-brain barrier permeability was examined 24 h after the ICH surgery through evans blue extravasation as described before (Manaenko et al., 2011). Briefly, an I.P. injection of 2% evans blue dye was given to the mice 3 h before a transcardial perfusion with 100 ml PBS. The brain was separated into right and left hemisphere, frozen with liquid nitrogen, and stored at −80 °C. 1000 μl of PBS was added to the right hemisphere tissue, which was homogenized and sonicated. The mixture was then centrifuged for 30 min at 14000 rpm and 4 °C. A mixture comprised of 500 μl of supernatant and 500 μl of 50% Trichloroacetic acid (TCA) was created and left to incubate at 4 °C overnight before centrifuging again. The resulting mixture was then

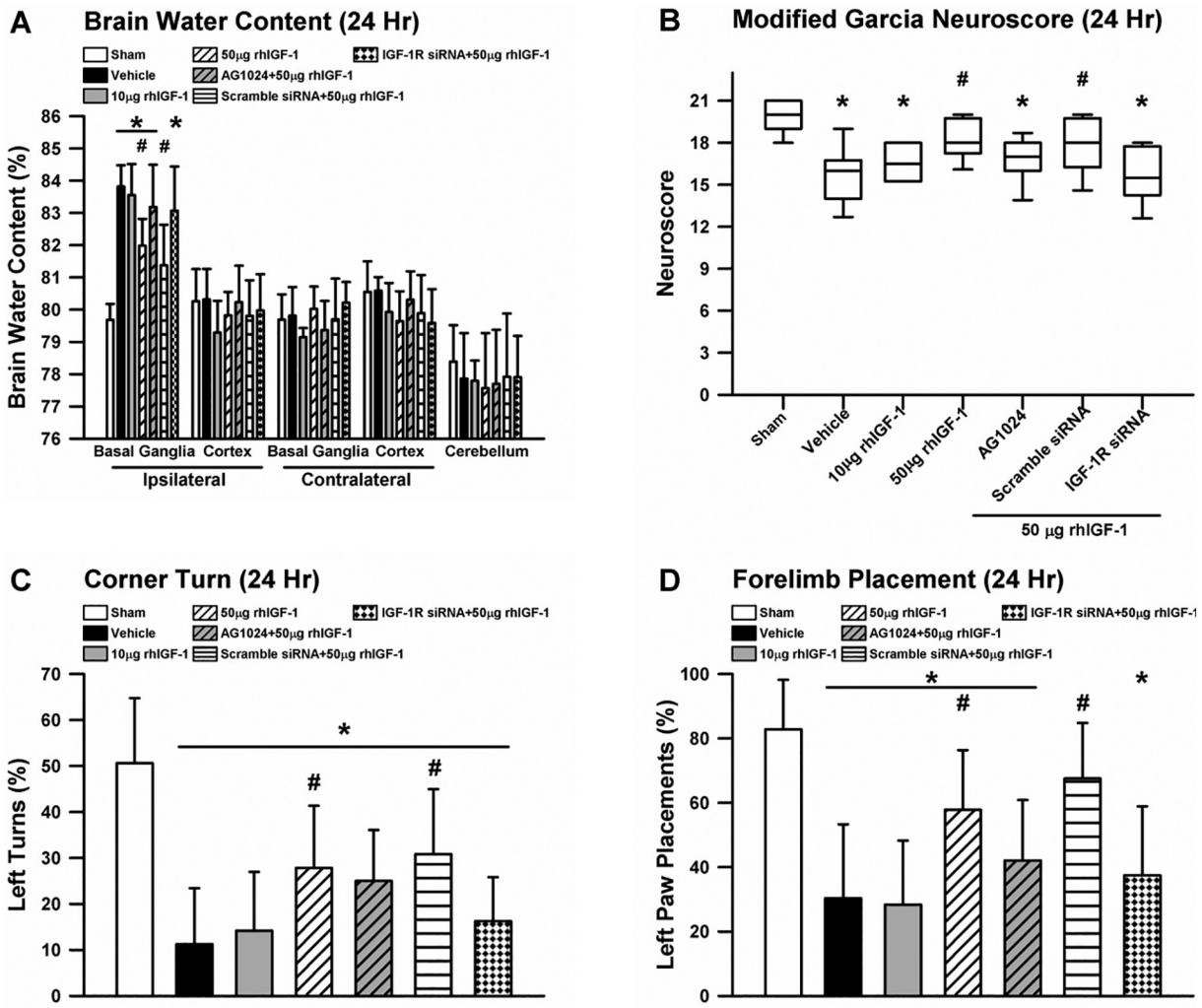


**Fig. 1.** Collagenase injection ICH model at 24 h. (A) Brain water content at 24 h (N = 6 per group). (B) Modified Garcia neuroscore. (C) Corner turn test. (D) Forelimb placement test. (B, C, D: N = 18 for Sham, Vehicle, and 50 μg rhIGF-1 groups; N = 12 for 10 μg rhIGF-1, AG1024 + 50 μg rhIGF-1, Scramble siRNA +50 μg rhIGF-1, and IGF-1R siRNA +50 μg rhIGF-1 groups). A, C, D: Values are expressed as mean ± SD. B: Values expressed as median ± IQR. *P < 0.05 compared with sham, #P < 0.05 compared with vehicle.

transferred to the spectrophotometer for analysis. A standard curve was created to analyze the data. The data is represented as μg of Evans Blue Dye/g of tissue.

### 2.8. Hemoglobin assay

For hematoma evaluation, the blood volume in the brain was examined using a hemoglobin assay (Manaenko et al., 2011). Briefly, 200 μl of the supernatant collected from the Evans Blue method described above was mixed with 800 μl of Drabkins's reagent (Sigma Aldrich) and left to incubate for 15 min at room temperature. The resulting mixture was then transferred to the spectrophotometer for analysis. A standard curve was created to analyze the data. Values are represented as μl of hemoglobin/hemisphere.

### 2.9. Western blotting

Animals were euthanized at 0, 3, 6, 12, 24, and 72 h for western blot analysis. At each time, animals were heavily sedated using isofluorane and euthanized through transcardial perfusions using 100 μl of PBS. The brain was divided into hemispheres and then frozen in liquid nitrogen and stored at −80 °C. The right and left hemispheres were removed from −80 °C storage and weighed before being homogenized in lysis buffer. After resting for 30 min, the samples were centrifuged for 20 min at 14000 rpm and 4 °C. The supernatant was collected, and the protein concentration was analyzed using a spectrophotometer. Western blots were done using the following primary antibodies. Anti-β-actin (1:2000), anti-p-GSK3β (Y216; 1:500), anti-GSK3β (1:500), anti-MEKK1 (1:500), anti-Occludin (1:1000), and anti-Claudin 5 (1:1000) were purchased from Abcam Inc. (Cambridge, MA). Anti-IGF-1 and anti-IGF-1R were purchased from Santa Cruz Biotechnology. (Santa Cruz, CA). The optical bands were visualized using ECL Plus (GE Healthcare Life Sciences) and analyzed with Image J Software (National Institute of Health).

### 2.10. Statistical evaluations

Data was expressed as mean ± Standard Deviation (SD) for brain water content, corner turn, forelimb placement, Evans blue, hemoglobin assay, and western blots. Significance analysis between groups was calculated using a One-Way Analysis of Variance with a Tukey post-hoc. Data for Modified Garcia neuroscore was expressed as median ± Interquartile Range (IQR). Significance analysis between groups was calculated using a Kruskal-Wallis One-Way Analysis on Ranks with Dunn's post-hoc test. Significance was indicated with a P value of < 0.05, unless otherwise noted. Statistical power was

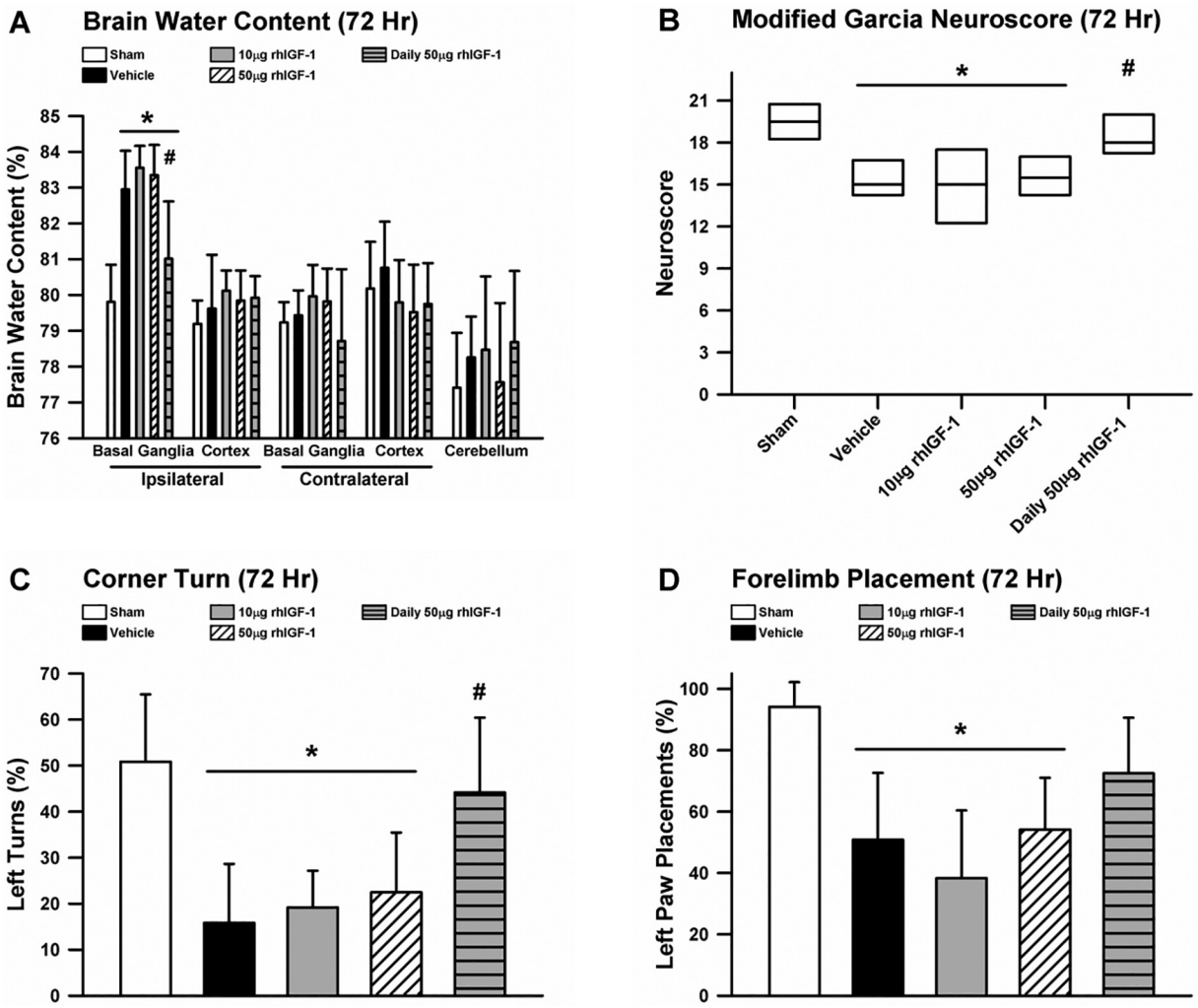


**Fig. 2.** Collagenase injection ICH model at 72 h. (A) Brain water content at 72 h (N = 6 per group). (B) Modified Garcia neuroscore (N = 6 per group). (C) Corner turn test (N = 6 per group). (D) Forelimb placement test (N = 6 per group). A, C, D: Values are expressed as mean ± SD. B: Values expressed as median ± IQR. *P < 0.05 compared with shame, #P < 0.05 compared with vehicle.

calculated for determining sample size using previously published data for brain water content in the ICH models before starting the study. Analysis was done using β = 0.80, α = 0.05, and a SD of 0.75. 6 mice were allocated to each group in the brain water content experiments with a minimal detectable difference in the means of 1.35. All statistics were generated using SigmaPlot version 11.0 (Systat Software).

## 3. Results

### *3.1. High dose of rhIGF-1 reduces brain edema and improves neurobehavior at 24 hours after collagenase induced ICH*

Brain water content (N = 6/group) and neurofunctional deficits (N = 12–18/group) were examined at 24 h following the surgery in the collagenase injection model. No animals were excluded from this experiment and no mortalities were found in any groups. All groups were compared to sham (* = P < 0.05) and vehicle (# = P < 0.05) groups. For brain water content, groups in tissue regions were compared independently. Mice subjected to ICH with vehicle, low dose (10 μg) of rhIGF-1, high dose (50 μg) of rhIGF-1, AG1024 with high dose of rhIGF-1, or IGF-1R siRNA with high dose of rhIGF-1 displayed significantly elevated brain water content compared with sham-operated animals in the ipsilateral basal ganglia (*p < 0.05; Fig. 1A). However, the high dose of rhIGF-1 and scramble siRNA with high dose of rhIGF-1 groups showed significantly decreased brain water content in the ipsilateral basal ganglia compared to vehicle (#p < 0.05, Fig. 1A). No significance was found with the scramble siRNA with rhIGF-1 group compared to sham (p > 0.05). No significant differences were calculated between groups for the ipsilateral cortex, contralateral cortex and basal ganglia, and the cerebellum (p > 0.05).

Mice subjected to ICH with vehicle (N = 18) showed significant neurobehavior deficits as compared to the sham (N = 18) operated animals (p < 0.05; Fig. 1B–D). The low dose of rhIGF-1 (N = 12) was unable to improve behavior scores in any test. However, the high dose of rhIGF-1 (N = 18) displayed an improvement to behavior scores as compared to the vehicle animals in all tests (p < 0.05; Fig. 1B–D), but remained significantly different from the sham group in the Corner Turn and Forelimb Placement tests (p < 0.05; Fig. 1C and D). The AG1024 (N = 12) and IGF-1R siRNA (N = 12) groups with the high dose of rhIGF-1 failed to demonstrate improved neurobehavior scores in all three tests (p > 0.05). The scramble siRNA (N = 12) group with the high dose of rhIGF-1 showed improved scores for all tests compared with vehicle (p < 0.05: Fig. 1B–D), but remained statistical significance different from the sham group in the Corner Turn Test (p < 0.05; Fig. 1C).

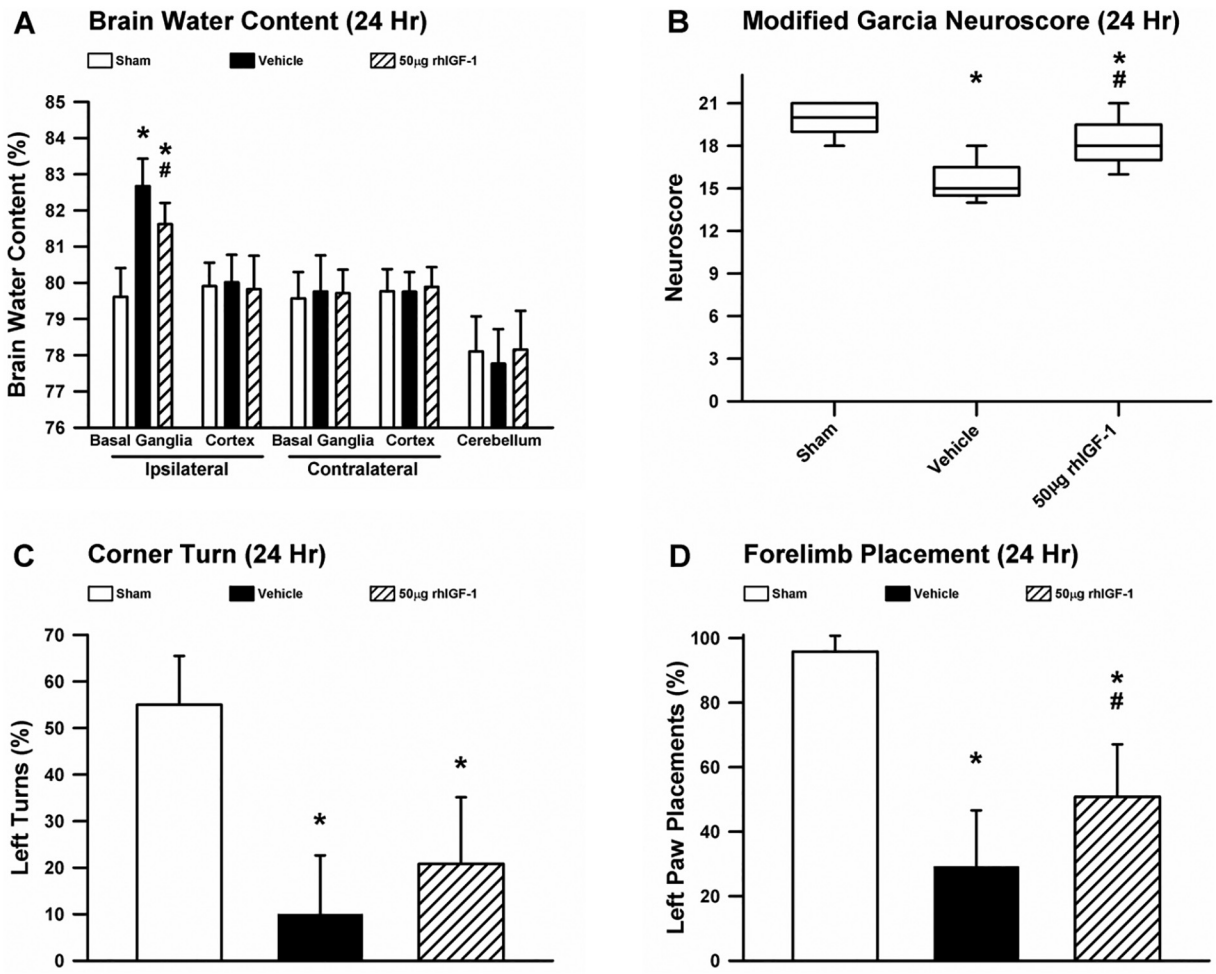


**Fig. 3.** Blood injection ICH model at 24 h. (A) Brain water content at 24 h (N = 6 per group). (B) Modified Garcia neuroscore (N = 6 per group). (C) Corner turn test (N = 6 per group). (D) Forelimb placement test (N = 6 per group). A, C, D: Values are expressed as mean ± SD. B: Values expressed as median ± IQR. *P < 0.05 compared with sham, #P < 0.05 compared with vehicle.

### *3.2. Daily administration of 50 μg rhIGF-1 reduces brain edema and improves neurobehavior at 72 hours after collagenase induced ICH*

Brain water content (N = 6/group) and neurofunctional deficits (N = 6/group) were examined at 72 h following the surgery in the collagenase injection model. In this experiment, 1 mouse under the "ICH + Vehicle" group was omitted due to mortality during the surgery procedure. All groups displayed statistically significant elevated brain edema compared with sham-operated animals in the ipsilateral basal ganglia ($p < 0.05$; Fig. 2A). The high dose of rhIGF-1 given daily showed significantly decreased brain water content in the ipsilateral basal ganglia compared to vehicle ($p < 0.05$). No statistical differences were observed between groups for the ipsilateral cortex, contralateral cortex, basal ganglia, and cerebellum ($p > 0.05$). Within the ipsilateral cortex, 10 μg of rhIGF-1 displayed a slight elevation to edema compared to sham but did not reach significant differences ($p = 0.059$).

Mice subjected to ICH with vehicle, low dose of rhIGF-1, and high dose of rhIGF-1 showed significant neurobehavior deficits as compared to the sham operated animals ($p < 0.05$; Fig. 2B–D). However, the high dose of rhIGF-1 (50 μg) given daily displayed improved scores in the Modified Garcia Neuroscore and Corner Turn tests as compared to the vehicle animals ($p < 0.05$; Fig. 2B and C). The daily dose of rhIGF-1 group was not significant to sham or vehicle groups in the Forelilmb Placement test ($p > 0.05$; Fig. 2D).

### *3.3. High dose of rhIGF-1 reduces brain edema and improves neurobehavior at 24 hours after autologous blood induced ICH*

Brain water content (N = 6/group) and neurofunctional deficits (N = 6/group) were examined at 24 h following the surgery in the autologous blood injection model. No animals were excluded from this experiment and no mortalities were found in any groups. Mice subjected to ICH with vehicle displayed significantly elevated brain water content compared to sham-operated animals ($p < 0.05$; Fig. 3A). A mild improvement was found in the high dose of rhIGF-1 treatment group compared to the vehicle operated group ($p = 0.048$). Additionally, the rhIGF-1 remained significantly different to the sham group ($p < 0.05$). No statistically significant differences were calculated between groups for the ipsilateral cortex, contralateral cortex and basal ganglia, and the cerebellum ($p > 0.05$).

Mice subjected to ICH with vehicle showed significant neurobehavior deficits as compared to the sham-operated animals in all exams ($p < 0.05$; Fig. 3B–D). The high dose of rhIGF-1 displayed improved neurobehavior scores compared to vehicle in the Modified Garcia Neuroscore and Forelimb placement exam ($p < 0.05$; Fig. 3B, D). The high dose of rhIGF-1 failed to significantly improve scores for the Corner Turn test ($p > 0.05$; Fig. 3C).

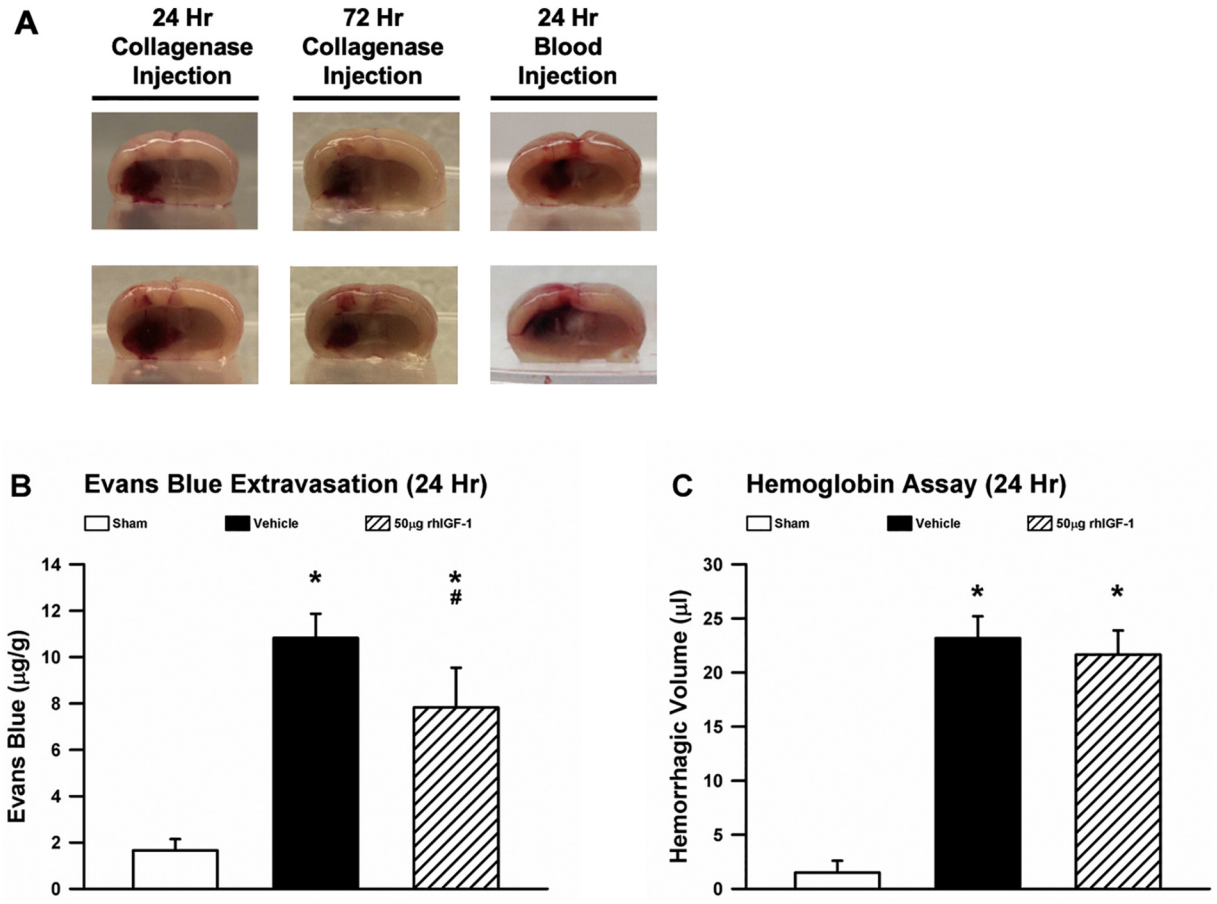


**Fig. 4.** (A) Representative images of hemorrhage location and size at 24 and 72 h for both collagenase injections and blood injections. (B) Evans blue extravasation at 24 h after collagenase injection (N = 6 per group). (C) Hemoglobin assay at 24 h after collagenase injection (N = 6 per group). Evans blue and Hemoglobin assay completed on ipsilateral hemisphere. Values are expressed as mean ± SD. *P < .05 compared with sham, #P < .05 compared with vehicle.

### *3.4. rhIGF-1 decreases blood-brain barrier permeability but does not change hematoma volume at 24 h after ICH*

Based on the results from the previous experiments measuring brain edema and neuroscores, the high dose of rhIGF-1 (50 μg) was used for the following studies. No animals were excluded from this experiment and no mortalities were found in any groups. Fig. 4A displays representative images of the hematoma formed in the collagenase induced ICH model at 24 and 72 h following the surgery and the autologous-blood induced ICH model at 24 h after the surgery. Animals from sham, vehicle, and rhIGF-1 treatment groups were tested for blood-brain barrier functionality at 24 h following the collagenase induced ICH surgery using Evans Blue Extravasation assays (N = 6/group). The ICH with vehicle group and rhIGF-1 group exhibited a statistically significant increase in Evans Blue content compared with sham animals in the ipsilateral hemispheric tissue ($p < 0.05$; Fig. 4B). However, rhIGF-1 significantly reduced the amount of Evans Blue present in the brain as compared to the vehicle group, thus suggesting a preserved BBB integrity ($p < 0.05$).

Hemoglobin assays were used to determine if changes to edema and BBB integrity outcomes were a result of a change in hematoma size (N = 6/group). ICH with vehicle animals showed a significantly greater amount of hemoglobin content as compared to the sham animals ($p < 0.05$, Fig. 4C). rhIGF-1 also showed significantly higher hemoglobin content than sham animals ($p < 0.05$), but failed to demonstrate a significant change as compared to vehicle ($p > 0.05$).

### *3.5. Endogenous IGF-1 but not IGF-1R expression is decreased following ICH*

Western Blot analyses of endogenous levels of IGF-1 and IGF-1R were examined in the ipsilateral brain tissue at 3, 6, 12, 24, and 72 h after the collagenase induced ICH surgery and compared to sham-operated tissue collected 24 h after the surgery (N = 6/group). No animals were excluded from this experiment and no mortalities were found in any groups. Changes in the expression of IGF-1 were found to exhibit an increase from 12 h to 72 h after the injury compared to sham results ($p < 0.05$; Fig. 5A). IGF-1R protein expression did not exhibit any significant changes at any time point compared to sham ($p > 0.05$; Fig. 5B).

### *3.6. rhIGF-1 reduces p-GSK3β and p-MEKK1 expression and increases Occludin and Claudin 5 expression*

Western Blot analyses of the ipsilateral brain hemisphere were conducted at 24 h following the collagenase induced ICH surgery (N = 6/group). In this experiment, 1 mouse under the "ICH + rhIGF-1 + scramble siRNA" group and 1 from the "ICH + rhIGF-1 + AG1024" group were omitted due to mortality during the surgery procedure. Sham and vehicle samples were shared from the previous experiment. Changes in the protein expression of p-GSK3β, MEKK1, Occludin, and Claudin 5 were quantified between groups (Fig. 6A–D). p-GSK3β was decreased in the rhIGF-1 and rhIGF-1 + scramble siRNA groups compared to the vehicle group ($p < 0.05$; Fig. 6A). AG1024 and IGF-1R siRNA increased expression of p-GSK3β compared to the sham group ($p < 0.05$). MEKK1 expression was decreased in the rhIGF-1, AG1024,

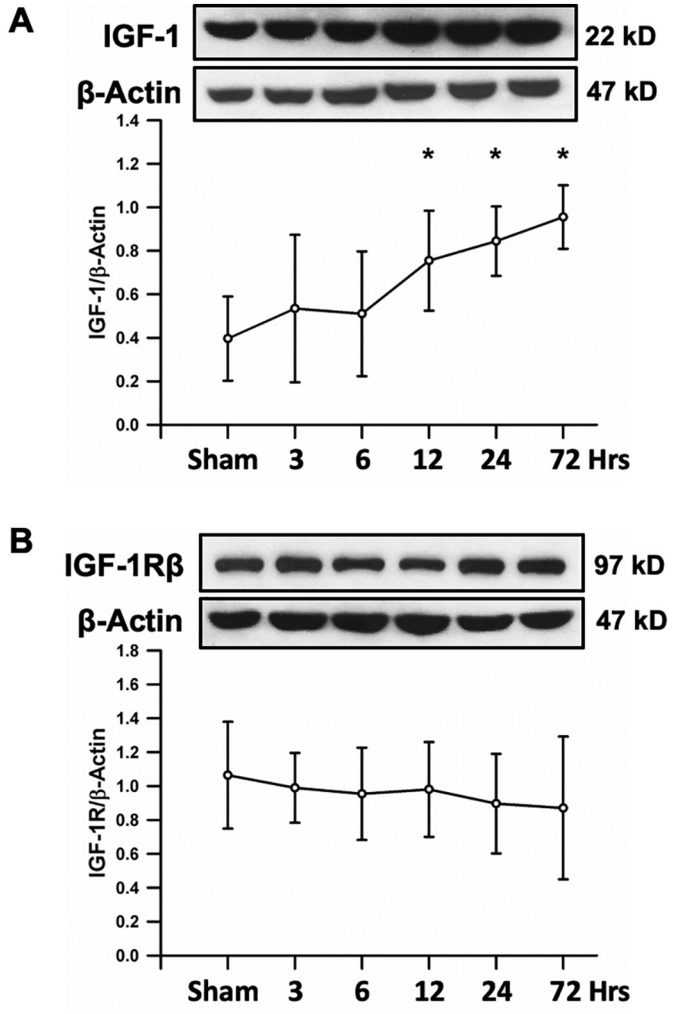


**Fig. 5.** Western blot analysis of (A) IGF-1 and (B) IGF-1Rβ. N = 6 per time point. Values are expressed as mean ± SD, normalized to sham. *P < 0.05 vs sham.

and scramble siRNA groups compared to the vehicle group ($p < 0.05$; Fig. 6B). IGF-1R siRNA elevated MEKK1 expression so it was no longer significant compared to the vehicle group ($p > 0.05$). Occludin and Claudin 5 expression was decreased in the vehicle, AG1024, and IGF-1R siRNA groups compared to the sham group ($p < 0.05$, Fig. 6C + D), whereas expression was elevated in the rhIGF-1 and rhIGF-1 + scramble siRNA groups so that it was no longer significant to sham ($p > 0.05$).

## 4. Discussion

The first aim of this study was to investigate if IGF-1R agonist rhIGF-1 could ameliorate brain edema and behavioral deficits after experimental ICH in mice. It is well known that basal ganglia hemorrhages lead to debilitating neurofunctional deficits in humans (Nys et al., 2007). The high dose of rhIGF-1 (50 μg) significantly improved edema and neurobehavior outcomes in the Garcia, corner turn, and forelimb placement test at 24 h after surgery (compared to vehicle) in the collagenase injection model while showing improvements in edema and Garcia and forelimb placement tests in the blood injection model. Interestingly, both doses of rhIGF-1 were unable to improve edema or neurobehavior at 72 h in the collagenase injection model. However, giving a daily dose of 50 μg of rhIGF-1 did show improvements 72 h after ICH. The half-life of rhIGF-1 has been previously reported to be 10–12 h and may suggest that the continued injury of ICH can overpower a single dose beyond the initial 24 h (Fouque et al., 1995). This may help to explain our observations and this data suggests that a continued treatment regimen may be needed for long-term studies on the use of rhIGF-1 on neuroprotection.

Our second objective was to determine if rhIGF-1 stimulation would decrease MEKK1/GSK3β activation thus decreasing BBB injury. Few experimental studies have evaluated the effects of IGF-1 treatment after ischemic stroke, but little information is given to its effect after a hemorrhagic stroke (Fletcher et al., 2009; Cai et al., 2011; Endres et al., 2007; Lin et al., 2009; Liu et al., 2001). Additionally, the role of IGF-1 in BBB preservation is not well documented, but few studies suggest a protective effect (Bake et al., 2016; Sharma and Johanson, 2007; Li et al., 1998). The improvement to edema by rhIGF-1 provides the initial evidence in this study to suggest that rhIGF-1 is able to stimulate the protection of the BBB. To further add support, we found that rhIGF-1 was also able to decrease permeability of the BBB through Evans blue extravasation (Fig. 4). Indeed, a previous study of ours showed that rhIGF-1 decreased BBB permeability in a germinal matrix hemorrhage model (Lekic et al., 2016). rhIGF-1 treatments did not, however, have any effect on the hematoma volume. This suggests that the injury model is stable, and the amount of bleeding is not affected by the treatment. Since the bleeding remains consistent between the groups, this indicates that the decrease in edema and Evans blue amount is a result of BBB permeability changes and not a result due to the amount of blood or severity of the injury.

The IGF-1R is located ubiquitously in several cell types including BBB forming endothelial cells (Chisalita and Arnqvist, 2004; Torres-Aleman et al., 1990). Stimulation of the IGF-1R has been shown to activate PI3K-Akt signaling (Bondy and Cheng, 2004). Additionally, Akt has been reported to reduce MEKK1 and GSK3β activation (Krafft et al., 2012; Han et al., 2006). GSK3β activation (phosphorylation at Tyr216) has been correlated to loss of tight junction expression including occludin and claudin-5 (Krafft et al., 2013; Ramirez et al., 2013). In connection with this theory, we found that there was greater p-GSK3β and MEKK1 expression and decreased occludin and claudin-5 expression in the brains of ICH animals, but that rhIGF-1 administration reversed these levels.

In order to ensure stimulation of the IGF-1R produced beneficial effects through this pathway, we administered AG1024 (a pharmaceutical competitive inhibitor of the receptor) and IGF-1R siRNA (to decrease the availability of the receptor) to try preventing the effects of the rhIGF-1 treatments (Parrizas et al., 1997; Luey and May, 2016). Both interventions reversed the beneficial effects on edema and neurobehavior by rhIGF-1 at 24 h in the collagenase injection model. Additionally, both also inhibited the effects of rhIGF-1 on protein expression levels of p-GSK3β, occludin, and claudin 5. However, AG1024 was unable to reverse the effect on MEKK1. Several reasons may explain why AG1024 was unable to change the levels of MEKK1 significantly. First, it could be that MEKK1 is not a major signaling molecule in the pathway we initially hypothesized. MEKK1 is a protein that is part of a signaling pathway activated by p-GSK3β and is involved in the activation of JNK and NFκB pathways (Lin et al., 2018; Morita et al., 2015). However, GSK3β has been demonstrated to signal several other downstream pathways that may have led to the change in occludin and claudin proteins (Ramirez et al., 2013; Xiao et al., 2017; Zhang et al., 2017; Severson et al., 2010; Hwang et al., 2018). However, because IGF-1R siRNA was able to change the levels of MEKK1, this suggests there is a relationship with IGF-1R stimulation and MEKK1. Another possibility is that the toxic environment from the hemorrhage produced may prevent AG1024 from fully being able to produce its effect. IGF-1R siRNA was given 24 h before the induction of the hemorrhage to decrease the available receptors that signaling can occur through, while AG1024 was given after the hemorrhage has been induced. Further studies are needed to determine the pathway that rhIGF-1 may confer its protective effects.

Interestingly, though we demonstrated that the high dose of rhIGF-1

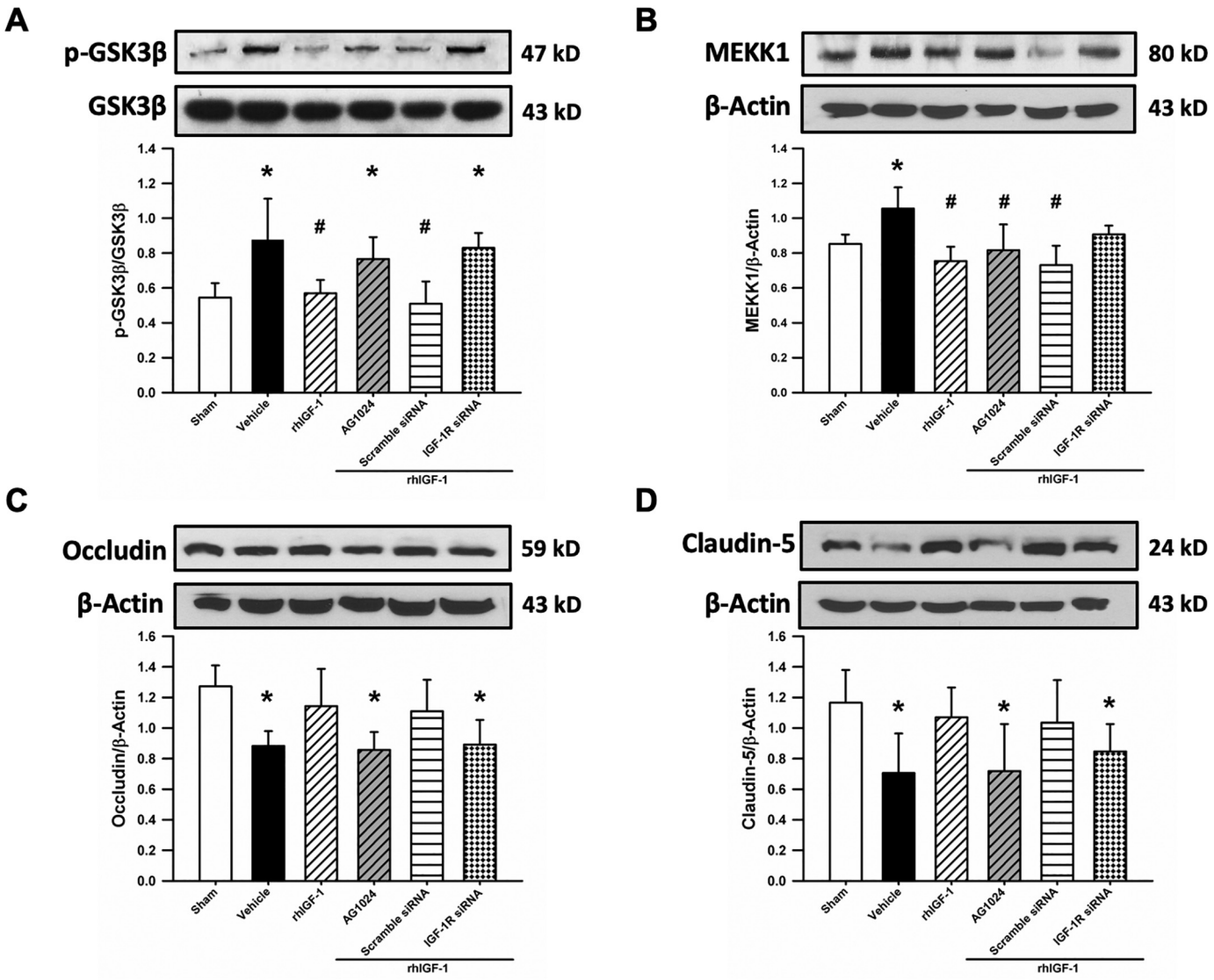


**Fig. 6.** Western blot analysis of (A) p-GSK3β, (B) MEKK1, (C) Occludin, and (D) Claudin 5. Values are expressed as mean ± SD, normalized to sham. *P < 0.05 compared with sham, #P < 0.05 compared with vehicle. N = 6 per group.

improves edema and neurofunctional outcomes at 24 h in the collagenase injection model, the protein expression of MEKK1 and occludin were only mildly improved. Additionally, there was only a mild improvement of edema and behavior in the blood injection model. Several factors can contribute to these results. First, rhIGF-1 may also effect cytotoxic edema (Johnston et al., 1996). We utilized both brain water content and Evans blue extravasation to evaluate BBB integrity. While brain water content can account for both cytotoxic and vasogenic brain edema, the Evans blue assay only observes BBB permeability through means of vasogenic brain edema (Carrozzino et al., 2009; Radu and Chernoff, 2013). It is generally accepted that both forms of brain edema are present 24 h after ICH (Yang et al., 1994). Second, the pathophysiological mechanisms of edema formation may be different between the collagenase and blood injection models. The collagenases infused are proteolytic enzymes that may have a greater effect on the BBB than the autologous blood (MacLellan et al., 2008). In addition, the edema formed from the blood injection model may also be more associated from the clot retraction causing serum extrusion (Mracsko and Veltkamp, 2014; Siaw-Debrah et al., 2017). As the mechanism of IGF-1 protection in this study is more appropriate to treating vasogenic edema, this may account for the milder improvements observed in the blood injection model. Third, treatments with rhIGF-1 may also improve outcomes through mechanisms of anti-inflammation and anti-apoptosis, which were not evaluated in this study (Cai et al., 2011; Spies et al., 2001; Wang et al., 2016; Hou et al., 2017). Finally, this may be a result of a difference in the sample sizes. Though the N values were consistent between the brain water content experiments, the changes in the sample size varied in the neurobehavioral outcomes as more experiments were done using the collagenase model.

Previous ischemic studies have shown that IGF-1 treatments can reduce the injury development promoting its use as a potential clinical treatment (Cai et al., 2011; Chang et al., 2011). However, some debate exists if IGF-1 will cross the blood-brain barrier effectively to exert its role on the cerebral tissues. Previous studies have therefore utilized the intranasal administration route to effectively deliver the drug to the brain (Fletcher et al., 2009). Utilizing this method, it was shown to effectively cross-the blood-brain barrier and protect against neuronal apoptosis. Though our study aims to study the blood-brain barrier and we would have no need to directly bypass it, we also applied the intranasal administration method to provide the maximum effectiveness to the cerebral tissues. We decided to use 50 μg of rhIGF-1 due to previous studies in neuroprotection showing positive effects using this dose (Cai et al., 2011). Being wary of the tumorigenic properties that high levels of IGF-1 can stimulate, we decided to observe if a lower dose

(10 μg) can also stimulate the neuroprotective qualities as lower doses have shown positive effects in other models of brain injury (Fletcher et al., 2009). Our recording of physiological parameters did not show significant variations to temperature and heart rate with administration of rhIGF-1, AG1024, scramble siRNA, or IGF-1R siRNA (data not shown).

Some limitations exist for this study that suggest a need for further studies. Though we were able to show that 50 μg of rhIGF-1 was able to show a certain level of neuroprotection, it still showed statistical significance in comparison to sham in several of the experiments including brain water content, neurobehavioral assessments, and Evans blue extravasation. Though Evans blue extravasation is a marker for blood-brain barrier permeability, it examines permeability based on the size of the openings that allow for the dye to infiltrate the brain (Saunders et al., 2015). Newer techniques such as albumin extravasation and histological analysis using fluorescent markers may provide more insight (Kassner and Merali, 2015). Other histological studies are needed to determine if there was a neuroprotection of the other cell populations by rhIGF-1. Furthermore, this study only viewed rhIGF-1 treatments given up to 3 days daily. Though an improvement in edema and neurobehavior can be seen up through that time, it is uncertain if continued treatments beyond this period of time will continue to show benefits to edema or for other potential protective effects from conditions of apoptosis or inflammation.

In summary, IGF-1R agonist rhIGF-1 reversed ICH-produced edema and neurobehavior deficits in both collagenase and blood injection models of ICH. In addition, these outcomes were paralleled by improved BBB integrity and molecular elevation of occludin and claudin-5 expression. However, IGF-1R antagonism by AG1024 and IGF-1R siRNA reversed the treatment effects on edema, behavior, and molecular expression. Based on these findings, we conclude that IGF-1R induced activation of the PI3K-Akt pathway resulted in the inhibition of p-GSK3β and MEKK1, thereby increasing occludin and claudin-5 expression and preserving the BBB.

## Contributions

D.S.N. contributed to the design, data acquisition, data analysis, interpretation, and drafted the article.

D.M., B.J.D., and A.M., participated in data acquisition and interpretation. J.T. and J.H.Z contributed to the study conception and design, interpretation of the results, and editing. Funding for this research was supported by the NIH grants R01NS091042 awarded to J.H.Z. and P01NS082184 awarded to J.T.

## Declaration of conflicting interests

The authors do not have any conflicts of interests.

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