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JWH133, A CANNABINOID RECEPTOR-2 AGONIST, ATTENUATES NEUROLOGICAL DEFICITS AND BRAIN EDEMA AFTER EXPERIMENTAL INTRACEREBRAL HEMORRHAGE IN MICE

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Abstract: Intracerebral hemorrhage is a subtype of stroke which has the highest mortality and morbidity rates and currently has no cure. Cannabinoid 2 (CB2) receptor expression is up-regulated in neuronal injuries. CB2 receptor agonists were found to be neuroprotective in brain injuries including ischaemic and hemorrhagic stroke. This study was carried out to investigate the effects of cannabinoid 2 receptor (CB2R) activation by JWH133- a selective CB2R agonist – on intracerebral hemorrhage (ICH) induced blood-brain barrier disruption and edema formation. ICH was induced in experimental animals by collagenase injection which causes significant vascular disruption and concomitant increase in edema; animals were then treated with JWH133. Sixty CD-1 mice were randomly divided into sham, vehicle, and JWH133-treated groups (1 mg/kg and 10 mg/kg). Neurobehaviour, brain water content, Evans Blue dye extravasation, hemoglobin content, lung water content, and body weights post ICH were assessed. JWH133 treatment attenuated neurological deficits at 24 and 72 h post-ICH. The treatment also reduced brain water content and Evans blue dye extravasation but had no effect on hemoglobin content and lung water content. Administration of JWH133 treatment mitigated weight loss at 48 and 72 h after ICH. The reduction in brain water content and Evans blue dye extravasation indicate that CB2 receptor activation decreases blood-brain barrier disruption and brain edema, resulting in improved neurological functioning. This suggests that activation of the CB2 receptor by JWH133 is neuroprotective after ICH and may be a therapeutic target. Further study is needed to explore the mechanisms by which these effects occur.

Keywords: JWH133, cannabinoid receptor 2, intracerebral hemorrhage, edema, neurological deficit

Intracerebral hemorrhage, the stroke subtype with the highest mortality and morbidity rates, is associated with significant vascular disruption and blood-brain barrier (BBB) dysfunction, which in turn leads to edema and leukocyte extravasation (1). The breakdown of the BBB can be triggered by the inflammatory reactions of the cells in the neurovascular unit, as well as immune-endothelial cell interactions (2-5). The haematoma that forms after ICH creates a mass effect that may also lead to disintegration of the endothelial tight junctions of the BBB, resulting in capillary leakage and neurological deterioration (6, 7).

Neurogenic pulmonary edema (NPE) characterized by acute onset of pulmonary edema is a potentially life-threatening syndrome resulting from CNS injuries such as intracerebral hemorrhage, subarachnoid hemorrhage, spinal cord injury, traumatic brain injury, intracranial hemorrhage, subdural hemorrhage, *status epilepticus,* and meningitis. NPE is thought to be due to a surge of catecholamines leading to cardiopulmonary dysfunction following neuronal injury. NPE though important in brain injury, is unpredictable, lacks specific diagnostic biomarkers and occurs sporadically, hence it is relatively 'underappreciated' and under-diagnosed clinically (8-11).

Cannabinoid (CB) receptors are G-protein coupled receptors that are activated by endogenous cannabinoids and synthetic tetrahydrocannabinol

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(THC)-like compounds (12). CB1 receptors are extensively distributed in the brain, while CB2 receptors (CB2R) are mainly expressed in activated astrocytes and microglia, neurons, central endothelial cells, and peripheral immune cells, and are inducible under inflammatory conditions. Expression of CB2R is also up-regulated in certain brain and spinal cord injuries (13-18).

Previous studies have demonstrated that CB2R activation is protective in both *in vivo* and *in vitro* models of cardiac ischemia, cerebral ischemia, subarachnoid hemorrhage, and neonatal germinal matrix hemorrhage (19-23). Activation of the CB2R has been shown to ameliorate centrally mediated pain responses and reduce inflammatory cytokines in a rat model of osteoarthritis (24). CB2R activation also reduced neuronal degeneration in Alzheimer's, disease Parkinson's disease and amyotrophic lateral sclerosis (25-28). Following ischaemic and reperfusion injury, administration of 0-1966, a selective CB2 receptor agonist reduced infarct volumes and improved memory and cognitive deficits (29). In *in-vitro* and isolated cell cultures, CB2R activation increased transendothelial electrical resistance and reduced induction of intercellular adhesion molecule and vascular cell adhesion molecule thereby reducing neuroinflammation and BBB permeability (30). CB2R activation was also found to reduce damage to the BBB by increasing the up-regulation of occludin and claudin 5 – tight junction proteins – responsible for maintaining the integrity of the BBB (30, 31). CB2R administration after subarachnoid hemorrhage reduced brain edema and neurological deficits by reducing brain infiltration of leukocytes (29, 32).

JWH133 is a selective CB2R agonist that has been shown to reduce infarct volumes and maintain the integrity of the BBB in rodent models of stroke (19, 21, 33). JWH 133 was also demonstrated to be neuroprotective in rats models of hemorrhagic stroke injury via amelioration of neuroinflammation and protection of blood-brain barrier integrity (34-37). In a model of subarachnoid hemorrhage, NPE was ameliorated by JWH133 (38).

Against this background, we hypothesized that activation of the CB2R by JWH133 would reduce the ICH-induced neurological deficits and neurogenic pulmonary edema by improving the integrity of the BBB and reducing brain edema.

Methods

All procedures were conducted in accordance with the NIH guide for the care and use of laboratory animals (39). Approval was obtained from the Institutional Animal Care and Use Committee of Loma Linda University. CD-1 mice weighing 29-38 g (Charles River, Wilmington, MA) were used for this study. They were kept in polypropylene cages and housed in temperature-controlled environment with 12 h of light and dark cycles. Experimental animals were given free access to food and water *ad libitum*.

Surgical procedures: ICH

Intracerebral hemorrhage was induced in mice by intrastriatal infusion of bacterial collagenase (40). Mice were injected intraperitoneally with 0.22 mg/kg Atropine. Anesthesia was achieved by intraperitoneal (IP) co-injection of 100 mg/kg Ketamine and 10 mg/kg Xylazine. Mice were pronated on a stereotactic head frame (Kopf Instruments, Tujunga CA). A 1 mm burr hole was drilled at these positions relative to bregma: 0.2 mm rostral and 2.2 mm right lateral. A 27-gauge needle (Microlitre No. 701; Hamilton Company, NV) was inserted 3.5 mm below the dura and 0.075 units of bacterial collagenase (Type VII-S, Sigma-Aldrich, St. Louis, MO), dissolved in 0.5 µL saline, was infused into the right basal ganglia at a rate of 0.1667 µl/min using a Namonite Syringe Pump (Harvard Apparatus, Holliston, MA). The needle was left for 5 minutes following complete injection of collagenase to prevent the backflow of bacterial collagenase up the needle tract. The needle was thereafter withdrawn slowly at a rate of 1mm/minute. Bone wax was used to seal the cranial burr hole, animals were treated with 0.4 mL of normal saline injected subcutaneously to minimize postsurgical dehydration. Experimental animals were allowed to fully recover from the surgical procedures under observation.

For sham-operated animals, the needle, without collagenase was injected into the right basal ganglia using the same coordinates and time frame as the other experimental animals.

Experimental groups and pharmacological interventions

Sixty animals randomly divided into the following groups were used for the study

- Sham (n = 18)
- ICH + Vehicle (n = 18)
- ICH + 1 mg JWH133 (n = 18)
- ICH + 10 mg JWH133 (n = 6).

Mice received JWH133 (in Tocrisolve™ 100, Tocris Bioscience, Minneapolis MN) or vehicle (Tocrisolve 100™ Tocris Bioscience, Minneapolis MN), via the intranasal route 1 hour after induction of ICH. Intranasal administration was achieved by

gently restraining each mouse, placing it in a supine position and administering drops of the drug or vehicle to each nare with the aid of a micro-pipette and micro-pipette tip. The total volume administered to each mouse was a maximum of 20 µL (41).

Assessment of neurological function

Behavioral outcomes were assessed at 24 and 72 h after ICH by observers blinded to the treatment administered to the various experimental animals.
The sensorimotor Garcia test is a 21-point assessment which evaluates 7 neurological indices: spontaneous activity, axial sensation, vibrissae proprioception, limb symmetry, lateral turning, forelimb outstretching, and climbing (42). Each test received a score between zero (worst performance) and 3 (best performance).

In the forelimb placement test, the animals were assessed for asymmetry in the sensorimotor striatum. This was done by holding the animals in such a way that their limbs hung freely. They were evaluated for their ability to reflexively move their forelimbs in response to stimulation of the whiskers (brushing against the edge of a table). The score was expressed as the number of successful *contra*lateral left paw placements out of a total of 10 consecutive vibrissae stimulations (43).

Mice were evaluated on their preference to turn left or right upon advancement into a 30° corner in the corner turn (CT) test. Animals with an injury will usually turn in one particular direction – the damaged side (*ipsi*lateral/right); hence, the test evaluates the magnitude of sensorimotor dysfunction. Choice of turning was recorded for a total of 10 trials and a score was calculated as the number of left turns/all trials × 100 (43, 44).

Brain water content (BWC) measurement

Brain water content was assayed at 24 and 72 h post ICH, as previously described (45, 46). Upon completion of neurobehavioural tests, mice were sacrificed by lethal isoflurane anesthesia, followed by decapitation and isolation of the brains. A 4 mm section, 2 mm each anterior and posterior to the needle tract, was separated and divided into *ipsi*lateral and contralateral cortices and basal ganglia. The cerebella were collected as an internal control for BWC of each tissue. The isolated tissue samples were all weighed separately using an analytical microbalance (APX-60 Denver Instrument, Bohemia, NY) to obtain wet weight (WW). They were then placed in an oven set to 100°C and left to dry for 24 and 72 h (to obtain constant weights) and the dry weights (DW) were then taken. Percentage BWC was calculated using the equation: (WW-DW)/WW × 100

Evans Blue extravasation

Evans Blue extravasation assays were conducted 24 h after ICH, as previously reported (47). Briefly, mice were restrained and given an intraperitoneal injection of 4 mL/kg of 4% Evans Blue dye. The mice were placed back into their cages for 3 h and then euthanized by trans-cardiac perfusion with phosphate-buffered saline (PBS). The right and left brain hemispheres were removed and placed in 1.5 mL tubes. Each hemisphere was homogenized in 1200 µL of PBS, sonicated, and centrifuged at 13000 rpm for 30 min. Trichloroacetic acid (500 µL) was added to each 500 µL aliquot of the supernatant. The mixture was incubated overnight at 4°C. It was then re-centrifuged and the extravasated Evans Blue dye quantified using a spectrophotometer at 610 nm (Thermo Fisher Scientific Inc. Waltham, MA). Results obtained are presented as µg of Evans Blue dye per g of brain tissue.

Hemoglobin assay

The spectrophotometric hemoglobin assay was performed as previously described (45, 48). Animals were perfused with PBS and the brains collected. *Ipsi*lateral and *contra*lateral hemispheres were homogenized separately in PBS, sonicated for 1 min, and centrifuged at 13000 rpm for 30 min. The supernatant was removed and transferred to fresh Eppendorf tubes. Drabkin's reagent (800 µL) was added to a 200 µL aliquot of the supernatant and the reaction mixtures were left to react for 15 min. The absorbance of hemoglobin was measured at 540 nm on a spectrophotometer and quantified using a standard curve.

Lung water content and body weight measurements

The lung water content measurement was obtained similar to the method of analyzing brain water content. After euthanizing the test animals, the lungs were removed from each animal and placed in pre-weighed 2 mL tubes. They were then weighed on an analytical microbalance (APX-60 Denver Instrument, Bohemia, NY) and the weight recorded as the wet weight. Samples were then placed in an oven and dried at 100°C for 24 h; the dry weight (DW) was then taken. Lung water content was calculated as (WW-DW)/WW × 100 (38).

Body weights were taken at prior to ICH and at the following time points post ICH-24, 48 and 72 h for the 24 and 72 h study respectively.

Statistical analysis

Data expressed as mean ± SEM was statistically analyzed by one-way ANOVA, followed by the Tukey post-hoc test. All behavior data were analyzed by one-way ANOVA on ranks, followed by Tukey post-hoc test. A probability value of less than 0.05 was considered statistically significant. All statistical analyses were performed using Sigma Plot version 11.0 for Windows.

RESULTS

Evaluation of brain water content and neurological outcomes at 24 h after ICH

Mean value for BWC in the *ipsi*lateral basal ganglia of sham-operated animals was 80.07 ± 0.23%, this was significantly different ($p < 0.05$) from vehicle (83.67 ± 0.33%), JWH133 1 mg/kg (81.70 ± 0.26%) and JWH133 10 mg/kg treated animals (81.62 ± 0.30%). BWC of vehicle-treated animals were also significantly different ($p < 0.05$) from those of JWH133 1 mg/kg and 10 mg/kg treated animals. No significant difference was seen in BWC of *ipsi*lateral cortex, *contra*lateral basal ganglia and cortex and the cerebella of sham-operated, vehicle and JWH133 treated animals. Data is presented in Figure 1A.

In the Garcia neurological test, sham-operated animals had higher scores (20.5 ± 0.34) which were significantly different ($p < 0.05$) from vehicle-treated animals (13.67 ± 0.42) and JWH133 1 and 10 mg/kg treated animals (16.50 ± 0.56 and 16.67 ± 0.49 respectively). Garcia neurological test scores of mice treated with 1 mg/kg and 10 mg/kg JWH133 were significantly different ($p < 0.05$) from those of vehicle-treated animals. Data is shown in Figure 2A. Sham operated animals had an average of 43.33 ± 3.33% left turns in the corner turn test while percentage left turns were 5.00 ± 3.41% and 6.67 ± 4.22% for vehicle and 10 mg/kg respectively. Percentage left turns of vehicle and 10 mg/kg were significantly different ($p < 0.05$) from sham animals. Percentage left turns for JWH133 at 1 mg/kg was 18.00 ± 4.19%; this was not significantly different from vehicle or sham-operated animals (Fig. 1C).

Figure 1D shows percentage scores from the left forelimb placement test. Sham-operated animals had an average of 98.33 ± 1.67% left forelimb placements. This was significantly different ($p < 0.05$) from the vehicle (33.33 ± 7.66%) and JWH133 1 and 10 mg/kg treatment group (51.67 ± 7.03 and 48.33 ± 6.01% respectively), though there was a tendency towards significance between forelimb placement values for vehicle and JWH133 treated animals.

Evaluation of brain water content and neurological outcomes at 72 h after ICH

As the low dose (1 mg) of JWH133 showed greater improvements at 24 h in the corner turn tests,

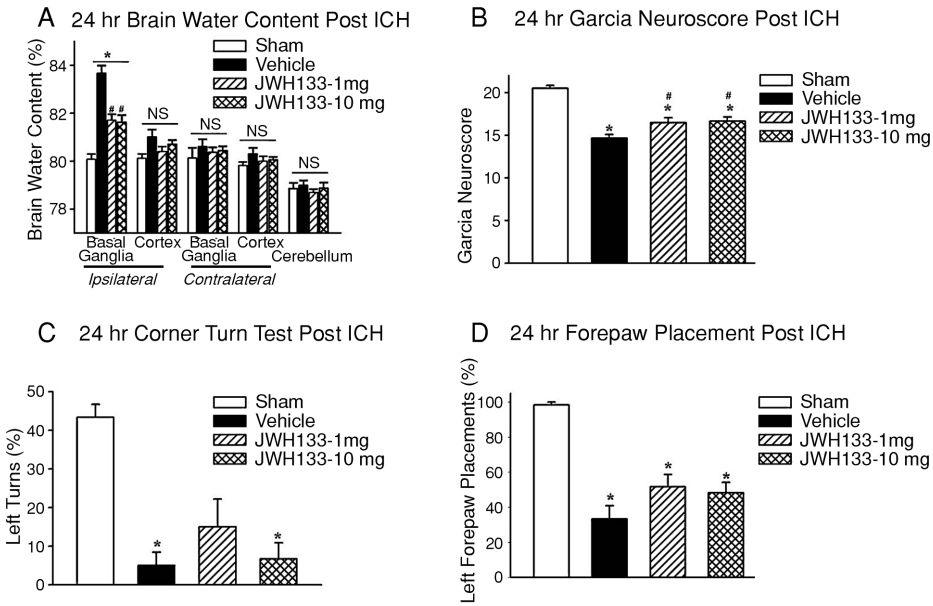

Figure 1. Brain water content and behavioral outcomes at 24 h after intracerebral haemorrhage induction or sham surgery. JWH133 (1 mg and 10 mg) reduced brain water content in *ipsi*lateral basal ganglia (A) and improved neurological deficits in the Garcia test (B). Only JWH133 (1mg) improved neurological deficits in the corner turn test (C). JWH133 (1 mg and 10 mg) improved neurological deficits in the forepaw placement test and had a tendency towards significant difference when compared to the vehicle treated-group (D). Data are-expressed as mean ± SEM. *$p < 0.05$ compared to sham, #$p < 0.05$ compared to vehicle. ICH indicates collagenase induced intracerebral haemorrhage. NS means not significant; n = 6 per group

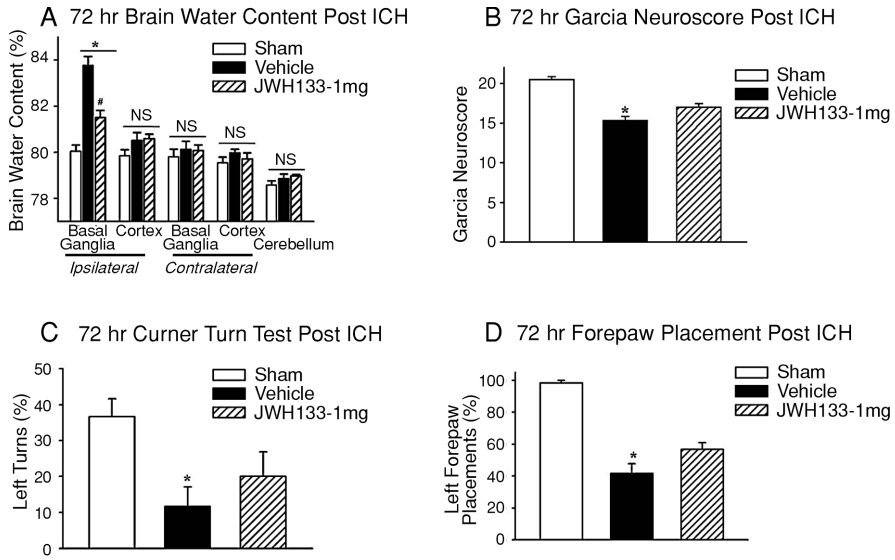

Figure 2. Brain water content and behavioral outcomes at 72 h after intracerebral haemorrhage induction or sham surgery. JWH133 (1 mg) reduced brain water content in *ipsi*lateral basal ganglia (A) and improved neurological deficits in the Garcia, corner turn and forepaw placement tests (B-D). Data are expressed as mean ± SEM. *p < 0.05 compared to sham, #p < 0.05 compared to vehicle, ICH indicates collagenase induced intracerebral haemorrhage. NS means not significant; n = 6 per group

it was chosen for the subsequent experiments. At 72 h after ICH, average BWC in sham-operated animals was (80.04 ± 0.27%); this was significantly different ($p < 0.05$) from vehicle-treated (83.76 ± 0.39%) and JWH133 treated animals (81.51 ± 0.30%). BWC in brains of JWH133 group was also significantly different ($p < 0.05$) from those of animals in the vehicle group. No significant difference was observed in the BWC of *ipsi*lateral cortex, *contra*lateral basal ganglia and cortex and the cerebella of sham-operated, vehicle and JWH133 treated animals. Data is presented in Fig. 2A.

In the Garcia neurological test, JWH improved test scores (17.00 ± 0.45). This was significantly different ($p < 0.05$) from vehicle-treated animals (15.33 ± 0.49) but not from sham-operated animals (20.50 ± 0.34). Data is presented in Figure 2B.

For the corner turn test, sham-operated animals gave a mean value of 36.67 ± 4.94, this was significantly different ($p < 0.05$) from mean values of the vehicle group (11.56 ± 5.43) but not significantly different from JWH133 (20.00 ± 6.83) treated animals (Fig. 2C).

In the forepaw placement test, sham animals gave an average score of 98.33 ± 1.61% which was significantly different ($p < 0.05$) from vehicle-treated animals (41.67 ± 6.04%) but not significantly different from JWH133 treated animals (55.67 ± 4.22). Data are shown in Figure 2D.

Evans Blue dye extravasation and hematoma volume after ICH

In the *ipsi*lateral hemisphere, Evans Blue leakage in sham-operated animals was 1.00 ± 0.30 µ/g of tissue, this was significantly different ($p < 0.05$) from values obtained from animals in the vehicle group (4.05 ± 0.37 µ/g) and JWH treated animals (2.09 ± 0.33 µ/g) Extravasated Evans Blue dye amount of measured in the *ipsi*lateral hemisphere of JWH133 group was significantly different ($p < 0.05$) from the vehicle-treated group (Fig. 3A).

No significant difference in hemorrhage volume was observed between vehicle and JWH133 treated (Fig. 3B).

Lung water content after ICH

There were no differences in lung water content after ICH in all the intervention groups (Fig. 4A).

Changes in body weight after ICH at 24, 48 and 72 h after ICH

At 24 h after ICH, no significant changes in body weights were observed in the sham-operated animals compared to baseline values. Baseline line values of body weights for vehicle, low and high dose JWH133 were significantly higher ($p < 0.05$) than weights recorded at 24 h post ICH. Data is presented in Figure 4B.

For the sham-operated animals, no changes in body weight were observed at 24, 48 and 72 h after ICH. In vehicle-treated animals, no improvement in weight loss was observed at 24, 48 and 72 h after ICH. In JWH treated animals, no improvement in body weight was seen and 24 h after ICH, however improvements which tended towards significant difference were noticeable by 48 and 72 h after ICH in the treatment groups (Fig. 4C).

DISCUSSION

Endocannabinoids (endogenous cannabinoids) mediate their actions mainly through the activation of two cannabinoid receptors: type 1 (CB1) and type 2 (CB2) (49). These play a major role in the pathophysiology of inflammatory diseases and have been found to be elevated in these conditions (50, 51). CB1 receptors are more widely expressed in the CNS, and their activation has been found to inhibit the release of proinflammatory cytokines while enhancing the release of anti-inflammatory cytokines (52, 53). CB2 receptors, on the other hand, are expressed in the periphery, particularly in the immune system and also in the central nervous system (54). However, they are inducible under certain pathological conditions, particularly inflammation-associated diseases, which make them attractive

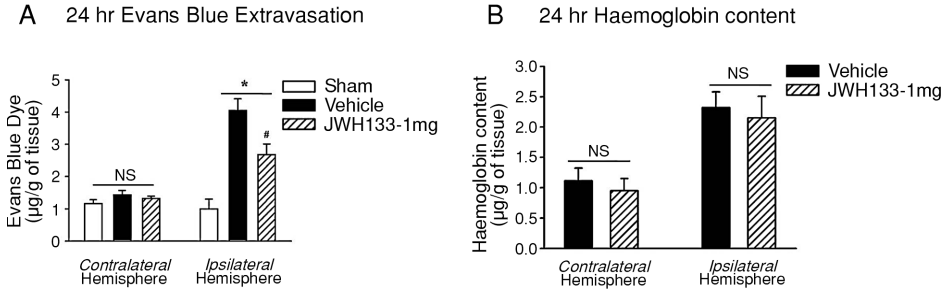

Figure 3. Evans Blue dye extravasation and hematoma volume (hemoglobin content) 24 h after ICH. JWH133 reduced the amount of extravasated dye in the *ipsi*lateral hemisphere (A) but did not reduce hematoma volume after ICH (B). Data are expressed as mean ± SEM. *p < 0.05 compared to sham, #p < 0.05 compared to Vehicle. NS means not significant; n = 6 per group

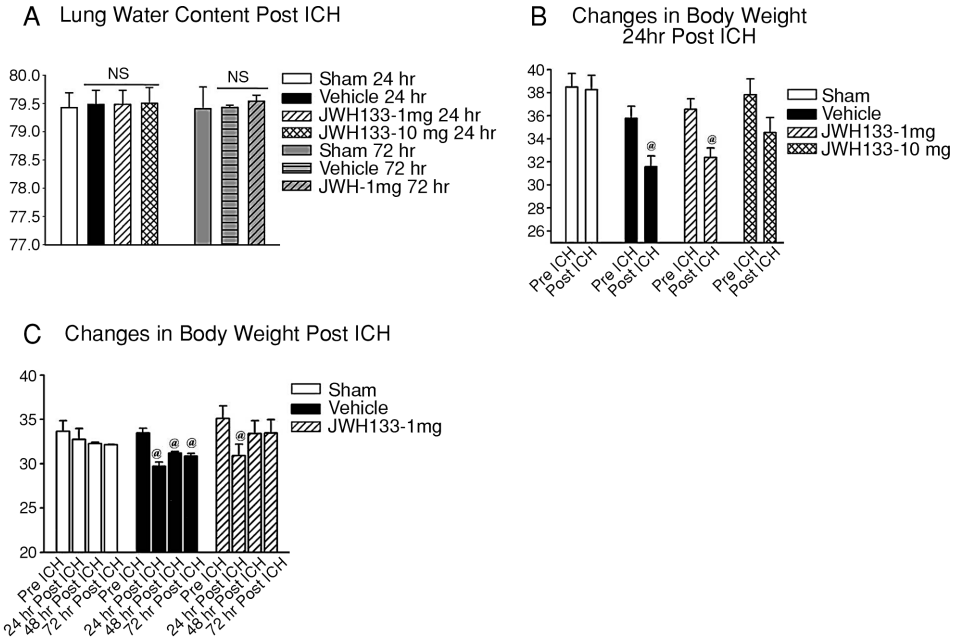

Figure 4. Lung water content (LWC) at 24 and 72 hours, and changes in body weight at 24, 48 and 72 h after ICH. JWH133 did not affect LWC after ICH (A). JWH133 failed to improve body weight at 24 h (B). An increase in body weight was noticeable at 48 and 72 h (C). Data are expressed as mean ± SEM. @ compared to baseline (Pre-ICH) body weight, NS means not significant; n = 6 per group

therapeutic targets (55-58), and recent research has been aimed at targeting the inflammatory immune response to stroke (59). ICH is associated with significant neuroinflammation and currently has no effective therapies, thus, targeting the CB2 receptor after ICH is highly relevant.

In a previous study, Fujii et al. reported that activation of CB2 receptors by JWH133 attenuated subarachnoid hemorrhage (SAH)-induced neurological deficits and edema, via suppressing leukocyte infiltration, thus protecting the BBB (60). Fujii and his colleagues also demonstrated that CB2 receptor activation significantly reduced SAH-induced apoptosis (14). Similar findings were reported in a germinal matrix hemorrhage (GMH) rat model, where activation of CB2 receptors led to improved neurological outcomes, and reduced neuroinflammation and edema (22,61). Furthermore, in GMH, CB2 receptor activation led to reduced microglial accumulation, thereby reducing inflammation-induced secondary brain injury (23). These findings indicate a significant protective role of CB2 receptors in brain injury, particularly in the context of hemorrhagic strokes. Hence, in this study, we aimed to investigate the role of CB2 receptors in attenuating ICH-induced neurological deficits. Activation of the CB2 receptor by the agonist JWH133 improved neurological deficits in the Garcia neurological and the corner turn tests and reduced ICH-induced brain edema and blood-brain barrier disruption. CB2 receptor activation also mitigated the ICH-induced weight loss at 48 and 72 h. However, it had no effect on hemoglobin or lung water content, compared to vehicle.

We first evaluated two different doses of JWH133 (1 mg/kg and 10 mg/kg). Both doses significantly reduced brain water content at 24 h post-ICH. However, in the neurobehavioural tests, mice treated with 1 mg/kg had greater improvements than the high dose in the corner turn tests. As such, only this dose was used for the 72 h outcome measurements. This corresponds with the existing literature, in which 1 mg/kg JWH133 treatment resulted in significant improvements in neurobehavioural function (21, 61). The reduced efficacy of the 10 mg/kg dose may be due to possible non-specific activation of the CB1 receptor at higher doses, although JWH133 has a 40-fold higher selectivity for the CB2 receptor (19, 61-62). This is of note as there are controversies on the effects of CB1 receptor activation, and its role in neuroprotection, since antagonizing the receptor in ischaemic stroke resulted in reduced infarct volumes and increased blood flow during occlusion (19, 64). Next, we determined the effect of CB2 receptor activation on ICH-induced brain water content. JWH133 resulted in significant edema reduction in the *ipsi*lateral hemisphere. Further, we examined if this decrease in edema was due to improved maintenance of BBB integrity; we found a significant reduction in Evans blue dye extravasation in the treatment group, suggesting that JWH133 improved the integrity of the BBB. In addition, because the hematoma can alter BBB integrity, we tested whether JWH133 was reducing BBB disruption via a reduction of the hematoma. However, we found no effects of the treatment on hemoglobin content indicating that JWH133 directly reduced BWC and improved BBB independent of hematoma expansion which occurs after ICH. These findings correspond with those of Li et al., (36) where activation of CB2 receptor after collagenase-induced ICH leads to attenuation of BBB disruption, reduction of inflammatory mediators and upregulation of tight junction proteins. Upregulation of tight junction proteins and increased expression of guanosine-5' -triphosphate-Rac1 pathway also resulted in improved BBB integrity and attenuation of ICH induced injury (37).

We also evaluated the effect of JWH133 on lung water content following ICH collagenase induced ICH. Suzuki et al., (65) demonstrated neurogenic pulmonary edema (shown as increased LWC) as a potentially life-threatening complication associated with subarachnoid hemorrhage. We, however, did not observe neurogenic pulmonary edema in this study as no significant difference in LWC occurred in the different intervention groups. Weight loss after stroke is thought to be associated with depression, dysphagia, or neurological deficits resulting in feeding difficulties (66, 67). Jonsson et al. reported significant weight loss in stroke patients and recommended monitoring of body weight post-injury (68). Treatment with JWH133 post collagenase induced ICH improved body weights in this study.

While we have found that CB2 receptor activation by JWH133 reduced BWC, ameliorated neurological deficits, improved BBB integrity and body weights, this study has some limitations viz:

- we did not measure the effect of JWH133 on the inflammatory cytokine profile.
- we did not quantify the protective effects of CB2 receptor activation on neuronal death in ICH.
- we could not elucidate the pathway by which CB2 receptors act after ICH, this would have increased our understanding of therapeutic targets to the CB2 receptor in the treatment of ICH.

CONCLUSION

CB2 receptor activation improved neurological outcomes after ICH, and reduced brain edema. Activation of the receptor also attenuated the disruption of the blood-brain barrier.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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