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The Potential Therapeutic Role Of Connexin Mimetic Peptide-43 In Traumatic Brain Injury (TBI)

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THE POTENTIAL THERAPEUTIC ROLE OF CONNEXIN MIMETIC PEPTIDE-43 IN TRAUMATIC BRAIN INJURY (TBI)

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ABSTRACT

Traumatic brain injury (TBI) is a serious public health concern accounting for a substantial sum of deaths and long-lasting neural deficits. Following TBI, a complex array of biochemical and neuroinflammatory cascades unfold, which can spread to distant areas of the brain relative to the focal point of the injury. The mechanisms of this spread of signalling molecules remains unclear. One hypothesis is that gap junctions aid in the quick dispersal. To determine the potential role of gap junctions in the spread of neuroinflammation and recovery post-TBI, a connexin mimetic peptide (CMP) was used to block gap junctions and the outcome of this treatment was analysed. The outcome following TBI for four treatment groups of vehicle (n=8), 5 µg/kg CMP (n=9), 10 µg/kg CMP (n=9), and 15 µg/kg CMP (n=9) were analysed using behaviour testing and histological analysis. Significant sensorimotor deficits were observed between baseline and post-TBI time points; however, no significant difference was observed between treatment groups. An analysis of contusion size demonstrated no significant sparing of cells in CMP treated animals, regardless of dose. The inflammatory response was investigated by measuring microglial activation using Iba-1 staining and comparing areas medial and ventral to the contusion with comparable uninjured areas in the contralateral hemisphere. As anticipated, a significant increase in microglial activation was observed; however, this increase was not affected by CMP treatment. A non-significant trend showed that groups receiving CMP treatment had worse outcomes in both behavioural and cellular analyses. Contrary to studies showing CMP benefits following spinal cord injury (SCI), this study did not demonstrate a potential therapeutic role for CMPs in TBI. This could potentially point to a different role for Gap junctions in TBI than in SCI or to different roles for the neuroinflammatory responses post injury.

INTRODUCTION

Traumatic brain injury (TBI) is a serious public health concern that accounts for a substantial sum of deaths and long-lasting neural deficits worldwide (CDC). Following TBI, a complex array of biochemical cascades unfold (Blennow, Hardy et al. 2012), many of which are not entirely understood. TBI results in both cell death and dysfunction. Primary cell death is local to the injury site and due to the mechanical force of the injury and local biochemical cascades that are activated (Mangiola, Vigo et al. 2015). It typically occurs in the most acute stage (within 24-48 hours) after the TBI. Secondary cell death spreads away from the focal point of impact, sometimes to remote areas in the brain, causing further damage to the brain via cascades of ongoing neuronal cell death (O'Carroll, Gorrie et al. 2013). This cell death process can be long lasting, up to months or years post injury (Muccigrosso, Ford et al. 2016). The mechanism through which this spread occurs or what causes cell death at secondary locations is yet to be specifically determined. Because acute primary cell death occurs so quickly following TBI, therapeutic targets focus more on secondary cell death pathways to prevent the spread of the injury.

It is known that, after TBI, there is a neuroinflammatory response in which activated microglia release a combination of pro- and anti-inflammatory molecules responsible for the inflammatory response of the brain (Witcher, Eiferman et al. 2015). Though this inflammation may be beneficial to the injured brain at first, the exaggerated release of these molecules (Lee-Kubli, Ingves et al. 2016), and prolonged inflammation may lead to more severe injury following TBI (Muccigrosso, Ford et al. 2016), especially when inflammation occurs in distant sections of the brain uninvolved in the primary injury site. Inflammatory molecules include cytokines and interleukins, which have a vast array of effects on surrounding tissues (Morganti-Kossmann, Raghupathi et al. 2012).

The quick and vast dispersal of these pro- and anti- inflammatory proteins by microglia is thought to be partially mediated via gap junctions between glial cells (Haghikia, Ladage et al. 2008). Gap junctions are structural channels formed by the trans-cellular interaction of two connexons. Each connexon is composed of six membrane proteins called connexins (Cx). Gap junctions are channels large enough to enable the quick intracellular transfer of a vast range of biological molecules via direct connection of cytosolic contents between adjacent cells (Prochnow 2014). These gap junctions are thought to contribute to secondary brain damage by enabling the spread of inflammation away from the primary injury site (Ohsumi, Nawashiro et al. 2010).

The potential role of connexins in stroke and spinal cord injury (SCI) has been established (Bennett, Garre et al. 2012, Huang, Han et al. 2012, O'Carroll, Gorrie et al. 2013, Lee-Kubli, Ingves et al. 2016), but their potential role in TBI is only recently emerging. Because many glial cells present in the brain express connexins as a means of intracellular cooperation, their potential role in cell-death post-TBI may provide insight into the turmoil that ensues after TBI and may also act, therefore, as a potential therapeutic target (Xie, Cui et al. 2015). Following TBI, there is an upregulation of connexin 43 (Cx43) surrounding the primary injury site (Cronin et al, 2008). Using a knock-out model, the downregulation of Cx43 post-TBI resulted in a neuroprotective effect (Chunlan Huang et al, 2012). Based on the evidence presented above, connexins may be a good therapeutic target to minimize the spread of neuroinflammation in the brain following TBI.

One way to block connexins is via connexin mimetic peptides (CMPs), connexin specific inhibitors that block gap junctions. CMPs for Cx43 have been used therapeutically in SCI and are proposed to work via blocking the spread of the inflammatory response to other parts of the central nervous system (CNS) (O'Carroll, Gorrie et al. 2013). In the O'Carroll study, a rodent model of SCI was used and CMPs were implanted one hour post-SCI via closed cortical impact (CCI) (12.5 mm drop, 10 g weight). Rats with CMPs showed an improvement in locomotor recovery compared to the control animals. This was also accompanied by a decreases in astrogliosis and microglial activation in adjacent sites to the primary lesion, which led to an increase in neuronal survival around the contusion site (O'Carroll et al, 2013).

Although connexins have been shown to potentially play a role in the spread of cell death and inflammation post-TBI, studies have not attempted to block connexins in a therapeutic approach. It is possible that by blocking gap junctions, secondary damage may be minimalised post TBI, which could lead to better recovery outcomes (Huang, Han et al. 2012). The current study aimed to elucidate the potential therapeutic utility of connexin mimetic peptide following a rodent model of TBI using controlled cortical impact (CCI) by measuring behaviour, contusion size, and the inflammatory response in the brain. If gap junctions do play a significant role in secondary cell death, then, hypothetically, the addition of CMPs following TBI will hinder the inflammatory response and improve both the behavioural and cellular outcomes.

MATERIALS AND METHODS

Animals

Male Fisher 344 rates (300-450g) were housed two per cage in the University of Auckland, New Zealand Animal Facility and were kept on a 12:12 hour light/dark cycle with food and water available *ad-libitum*. The animals were handled daily for approximately 1 week prior to surgery for habituation. All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Animals and were approved by the University of Auckland and DePaul Institutional Animal Care and Use Committee.

Rats were given a CCI and were randomly assigned into the following groups: Vehicle (no treatment, n = 8); Concentration 1 (5 µmol/kg, n = 9); Concentration 2 (10 µmol/kg, n = 9); and Concentration 3 (15 µmol/kg, n = 9).

Experimental Design

All animals were acclimated to handling 1 week prior to initial injuries. Baseline motor function tests were conducted prior to induction of the (CCI). Either the vehicle consisting of artificial cerebral spinal fluid or a concentration (5, 10, or 15 µmol/kg) of connexin-43 mimetic peptide was

administered to the surface of the brain within 30-45 minutes post CCI via a stereotaxically implanted cannula connected to an osmotic minipump (Alzet, Cupertino, CA).

All animals underwent behavioural testing on days 3 and 7 post-CCI. The Cylinder/Limb Use and Rotarod tests were performed to observe any motor deficits among the groups (see Table 1). Rats were sacrificed on day 7 and the brains were perfused for immunohistochemical analysis. Contusion size and microglial activation was measured and compared between the groups.

Cortical Contusion Injury (CCI)

The cortical contusion injury was administered using a modified procedure developed previously (Sutton, Lescaudron et al. 1993). Rats were anesthetised with sodium pentobarbital (60 mg/kg) administered intraperitoneally.

After induction of anaesthesia, the rate was administered a local analgesic (Marcaine; 1-2 mg/kg) subcutaneously under where the incision was made to expose the skull. Care was taken to ensure that at no point the Marcaine came into contact with the cells in the CNS and no adverse effect on the experimental procedure could occur.

Once anaesthetised, the rat's head was shaved and placed in a Kopf stereotaxic apparatus. The skull was exposed and the bite bar adjusted to level bregma and lambda in the horizontal plane. A 4-5 mm diameter craniotomy was placed 0.5 mm anterior or 4 mm lateral to bregma, directly over the forelimb sensorimotor cortex. The cortical impact was delivered via a controlled cortical impact injury device (CCI) called the Benchmark Impactor. The injury was produced by a flat and circular impactor tip on the exposed brain (3 mm diameter, 3.0 m/s, 18.0 degrees, 2.0-2.5 mm depth for 250 ms) based on a stereotaxic rat brain atlas [G. Paxinos, C. Watson, *The Rat Brain in Stereotaxic Coordinatesx 2nd ed.*, Academic Press (1986)].

Connexin Mimetic Peptide Infusion

Within the first 15-30 minutes post-CCI, using the standard stereotaxic surgical techniques described above, rodents were implanted with a brain infusion cannula (Alzet Brain Infusion Kit, ALZA Corporation) with a flow rate of 8 µL/hr connected to a mini osmotic pump (Alzet, Cupertino, CA) via catheter tubing. The pumps are designed for acute or chronic continuous infusions at a standard rate. The infusion cannula was placed onto the surface of the brain using the following stereotaxic coordinates (0.5 mm anterior and 4 mm lateral to bregma, and 0.8 mm ventral) and secured to the skull with tissue adhesive. The mini pump was placed in the subcutaneous space between the shoulder blades and the incision was closed with sutures. The infusion cannula was used to deliver either Cx43 mimetic peptide (5, 10, or 15 µmol/kg) vehicle solution (artificial cerebral spinal fluid) over a 24 hr period following injury. The pump remained n place until the rodent was killed 7 days post injury.

After the impact and implantation, Gelfoam was placed over the exposed brain. The rats were sutured with monofilament nylon sutures using a continuous suture of an interrupted pattern.

Following completion of the surgery, the rats received fluid replacement therapy (1 mL 0.9 % sterline saline per 100 g body weight) and post-operative analgesia (Emla cream) administered topically on the wound. A triple-antibiotic was applied to the wound at that time to prevent any topical infections.

Rats were returned to their home cage in the surgery room, which was placed on a heating pad. When they were mobile, their respiratory rate stabilized, and they demonstrated a righting reflex, they were returned to the vivarium. Upon recovery, the rats were housed 2 to a cage and monitored by the surgeon daily.

Rotarod Testing

Motor coordination and motor learning was assessed on an accelerating rotarod apaparatus (TSE Systems, Germany). Rotarod tested was conducted 1 day prior to receiving the CCI and on days 3 and 7 post-CCI. The rotarod was accelerated linearly from 4-40 rotations per minute (r.p.m) over 3 minutes. The latency to fall was recorded for each of the three trials by an experimenter blinded to group assignment.

Limb Use Testing

Asymmetries in forelimb use was assessed using the Schallert Cylinder Test. The animal was placed in a Plexiglass cylinder (GET Measurements) and the animal's behaviour was videotaped for 5 minutes. The videotapes were analysed for instances of either right, left, or both forelimb use for rearing, landing, and support along the walls of the cylinder. Data is presented as Percent ipsilateral forelimb use.

Euthanasia.

Animals were sacrificed 7 days following the CCI. Rats were deeply anesthetised with Equithesin (149 mg/100 g chloral hydrate, 31 mg/100 g sodium pentobarbital i.p.) prior to cardiac perfusion with 4 % buffered paraformaldehyde for histological preparations. The level of anaesthesia was assessed by the absence of reflex in response to toe pinch. Brains were extracted using phosphate buffered saline (PBS) with 0.005 % heparin and 4 % formaldehyde in PBS. Following extraction, brains were examined for any surface damage before being post-fixed, cryoprotected, and kept at 4 ºC until sectioning.

Measuring Contusion Size

Brains were flash-frozen and sliced serially in 40 µm coronal sections in sets of 7 using a cryostat. 1 set of tissues were mounted on gelatin-coated slides and stained with NeuN (monoclonal mouse anti-NeuN, Chemicon MAB377 1:1000; biotinylated horse anti mouse IgG, Vector BA2000 1:400), which stains most neurons using diaminobenzamide (DAB) and nickel sulphate forming the precipitate. Sections extended from interaural 11.70 mm, Bregma 2.70 mm to interaural 9.20 mm to Bregma 0.20 mm. The remaining cortical area around the contusion in the injured hemisphere was measured quantitatively using a Leica microscope (Leica Microsystems Inc,. Buffalo Grove, IL) with Neurolucida software. Cortical volume was obtained by multiplying the area by 240 µm, the distance between serial sets. The cortical area was measured in cubic micrometres.

Measuring Microglia Activation

1 set of tissue were mounted on gelatin-coated slides and stained with Iba-1 (polyclonal rabbit anti-Iba-1, Wako-019-19741-1:1500; biotinylated goat anti rabbit IgG, Vector BA1000 – 1:1400), which used an antibody against ionised calcium-binding adaptor molecule 1 (Iba1) with DAB and nickel sulphate forming the black precipitate. Iba1 is expressed normally by microglia in the brain but is upregulated by injury (D. Ito et al., 1998). An image of the medial and ventral cortical area to the contusion in the injured hemisphere was captured using Neurolucida software on the Leica microscope. The same areas on the contralateral hemisphere were also imaged for comparison. Sections with the same range as outlined in the contusion size method were used for the Iba-1 analysis. For each animal, three sections were chosen with two areas on each hemisphere for a total of twelve images per animal. A medial and ventral measurement was taken relative to the contusion on the injured hemisphere, while a measurement on the homotopic area of the contralateral uninjured hemisphere was used as a comparison. Ventral measurements were 500 µm x 500 µm while medial measurements were 250 µm width and 500 µm height.

Freely available ImageJ software (NIH) was used to process all of the images using a macro consisting of the following steps:

1. Convert to 8-bit image
2. Subtract background with a rolling ball radius of 50 pixels
3. Set threshold (30 – 255)
4. Convert to mask
5. Restore selection
6. Measure (measurements selected were area and area fraction; results were limited to threshold)

Results for Iba1 were reported as the area of immunoreactivity as a percentage of the total area of the cortex for each section and each area (medial or ventral) on the injured or uninjured hemisphere. The mean percentage area was calculated for each animal.

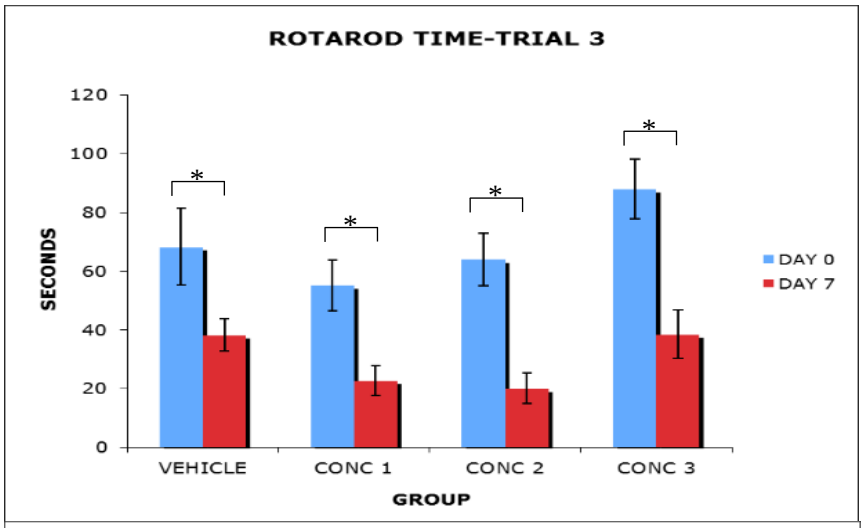
Statistical Analysis
Statistical analysis was performed using Vassarstats®. Two-way repeated measures ANOVA's were performed for both rotarod and limb use behaviour tests, while a one-way ANOVA was used on both immunohistological analyses. An alpha value of 0.05 adhered to on all tests. The Tukey Post-Hoc test was carried out following single factorial ANOVA testing.

DATA AND RESULTS

Rotarod Behavioural Testing at Day 1 and Day 7 Post-TBI

To investigate expected motor deficits and deficits in motor learning post-TBI, rotarod behavioural testing was conducted. As expected, motor deficits were observed between pre- and post-TBI. A significant difference was observed between time point day zero and day seven for both trial 3 time $F(1, 62) = 42.42$, $p < 0.0001$ and average speed $F(1, 62) = 43.1$, $p < 0.0001$). However, no significant difference was observed between treatment groups (Figure 1a-b).

1a



1b

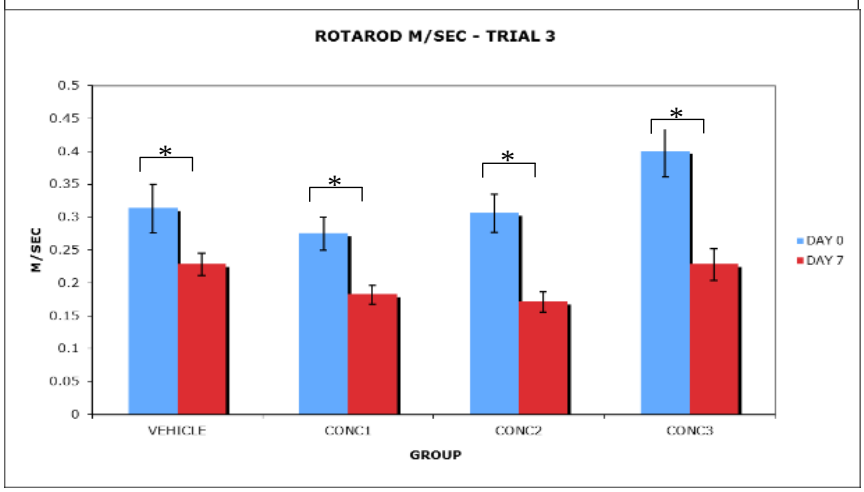


Figure 1
Presented rotarod testing data with two time points. (1a) Time travelled was observed between the treatment groups at day 0 and day 7. A significant difference was observed between time points but not between concentration groups. (1b) No significant difference was observed between the average speeds between treatment groups.

Limb Use Behavioural Testing

Forelimb use was analysed using standard limb use behaviour testing to detect the favouring of one limb over another during exploratory behaviours. Post-TBI, rats preferentially used the forelimb ipsilateral to the injury compared to baseline $F(1, 22) = 80.23$, $p < 0.0001$). However, no statistically significant differences were observed between the treatment groups (Fig. 2). A nonsignificant trend was observed demonstrating an increase in ipsilateral forelimb use between the vehicle and treatment groups.

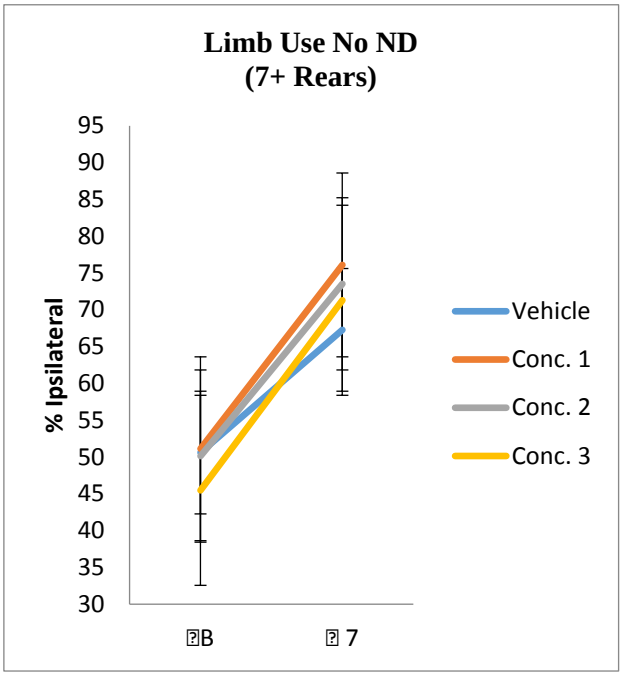
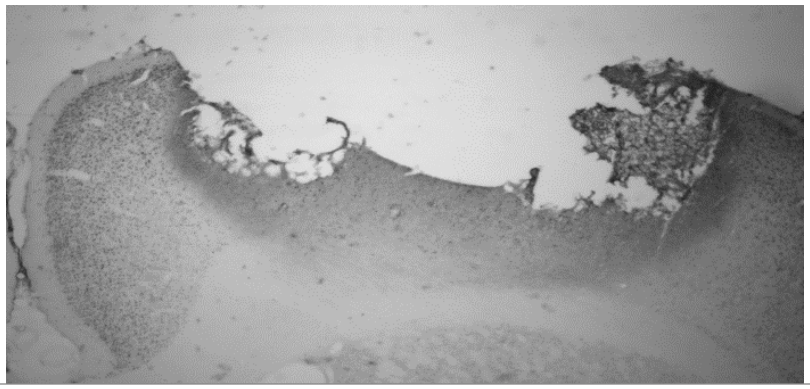


Figure 2
Limb use testing data for the four different treatment groups. No significant difference was observed between the four treatment groups. A non-significant trend is illustrated on the figure above, showing that treated groups performed worse than the vehicle. Each rat was counted only if it completed a total of 7+ rears.

Primary Injury Site and Contusion Size

The cortical area remaining in the injured hemisphere was analysed (Fig. 3a) and compared between the four treatment groups. The average contusion sizes between the four groups did not significantly change (Fig. 3b).

3a



3b

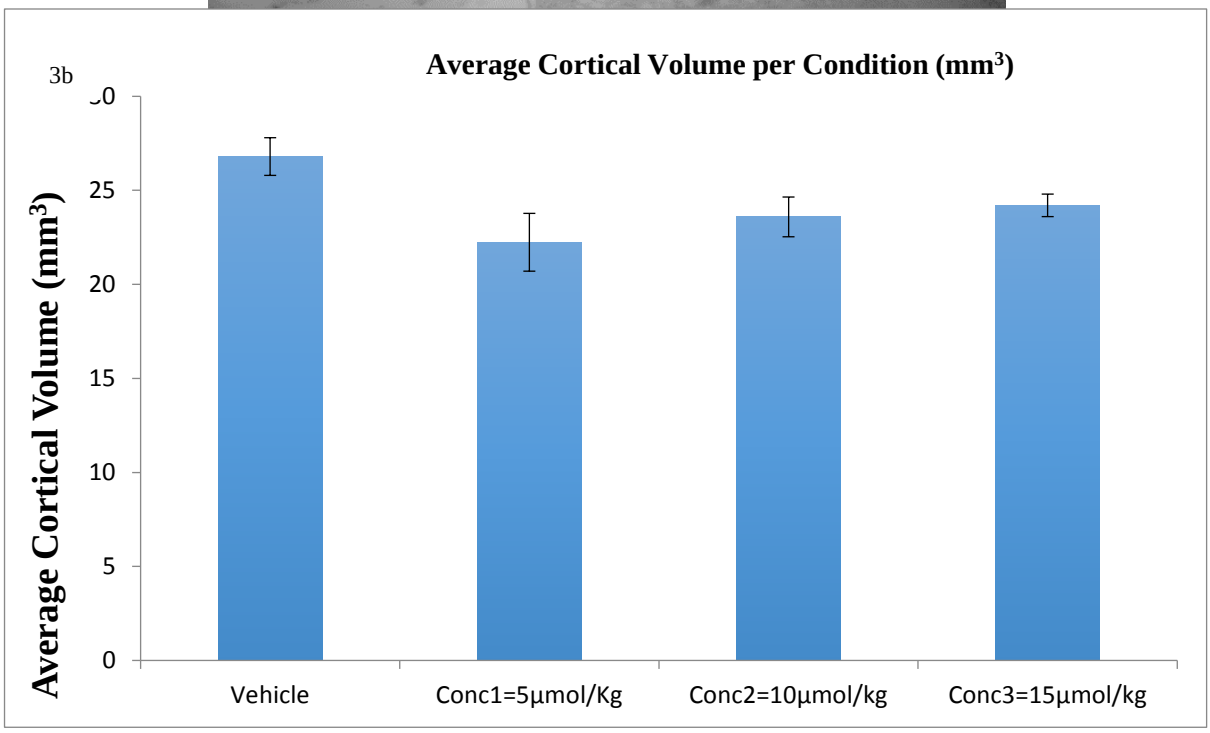


Figure 3
Average remaining cortical volume was measured surrounding the contusion site for all treatment groups. (3a) The area was traced using Neurolucida and multiplied by the depth of sections. (3b) No significant difference in remaining cortical area was observed between treatment groups ($p = 0.07$). However, a non-significant trend showed a decrease in average cortical area for groups receiving CMP.

Microglial Activation in the Injured Hemisphere and Contralateral Hemisphere

To explore microglial activation between the two hemispheres, images of four cortical areas were taken and analysed via densitometry for microglial densities (Fig. 4a-h). The spread of microglial activation was reported as a percent area occupied by the microglia in a predetermined area. As expected, a significant difference in microglial activation was observed between the injured hemisphere and the contralateral hemisphere for the medial areas $F(1, 188) = 249.02$, $p < 0.0001$ and the ventral areas $F(1, 176) = 303.73$, $p < 0.0001$. However, no significant difference was observed between the two groups (Fig 5a).

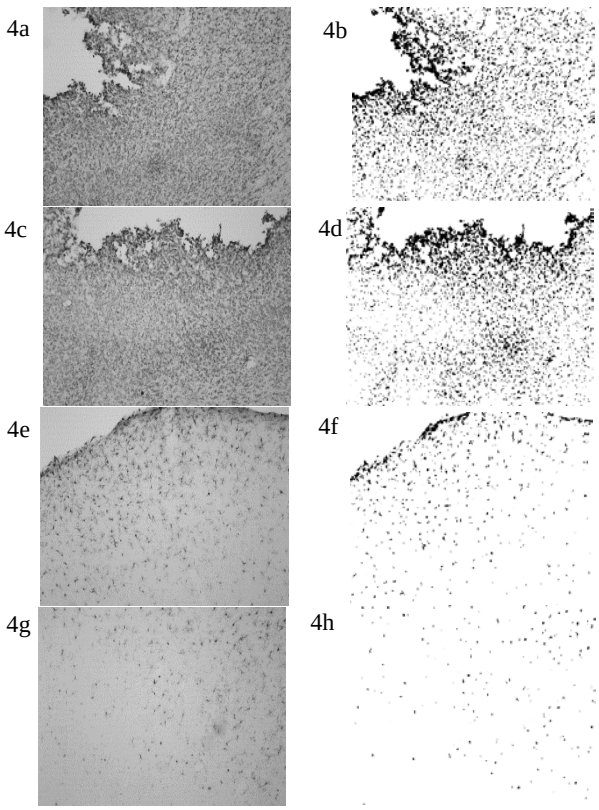
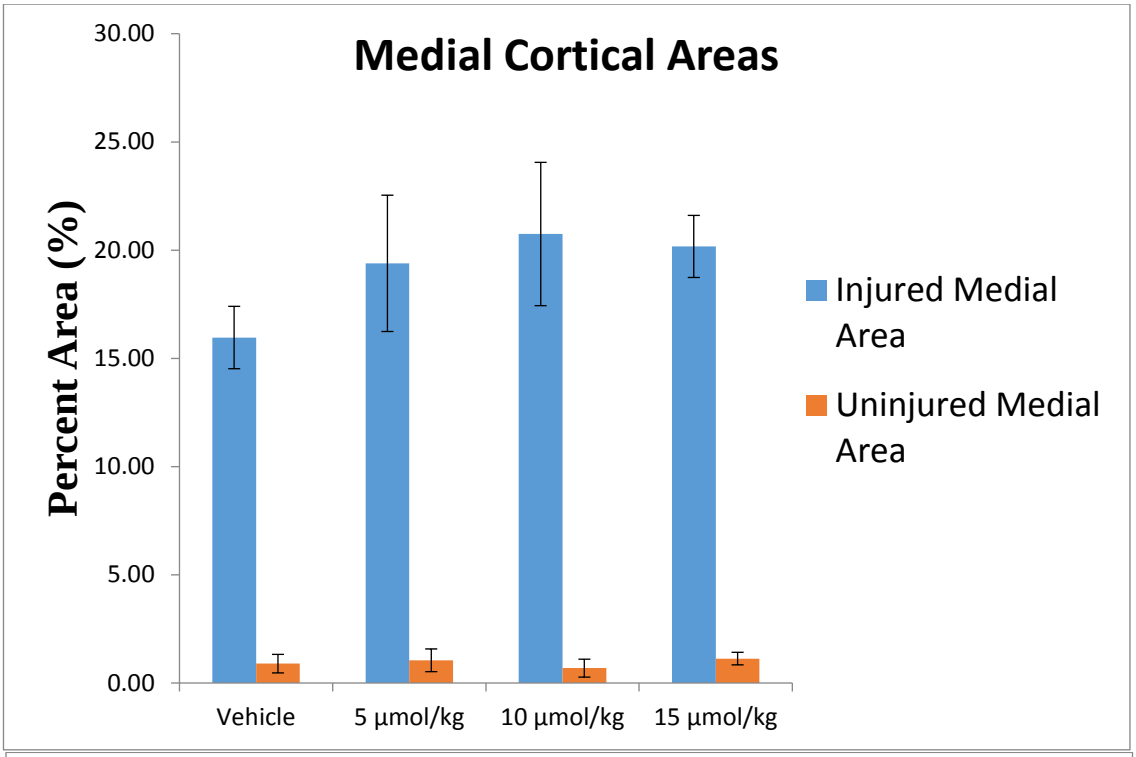


Figure 4
Analysis of microglial activation in four cortical areas, two on the injured and uninjured hemispheres. (4a) Medial area uninjured hemisphere. (4b) Medial area uninjured hemisphere processed through ImageJ. (4c) Ventral area injured hemisphere unprocessed. (4d) Ventral area injured hemisphere processed through ImageJ. (4e) Medial area uninjured hemisphere unprocessed. (4f) Medial area uninjured hemisphere processed through ImageJ. (4g) Ventral area uninjured hemisphere unprocessed. (4h) Ventral area uninjured hemisphere processed through ImageJ.

5a



5b

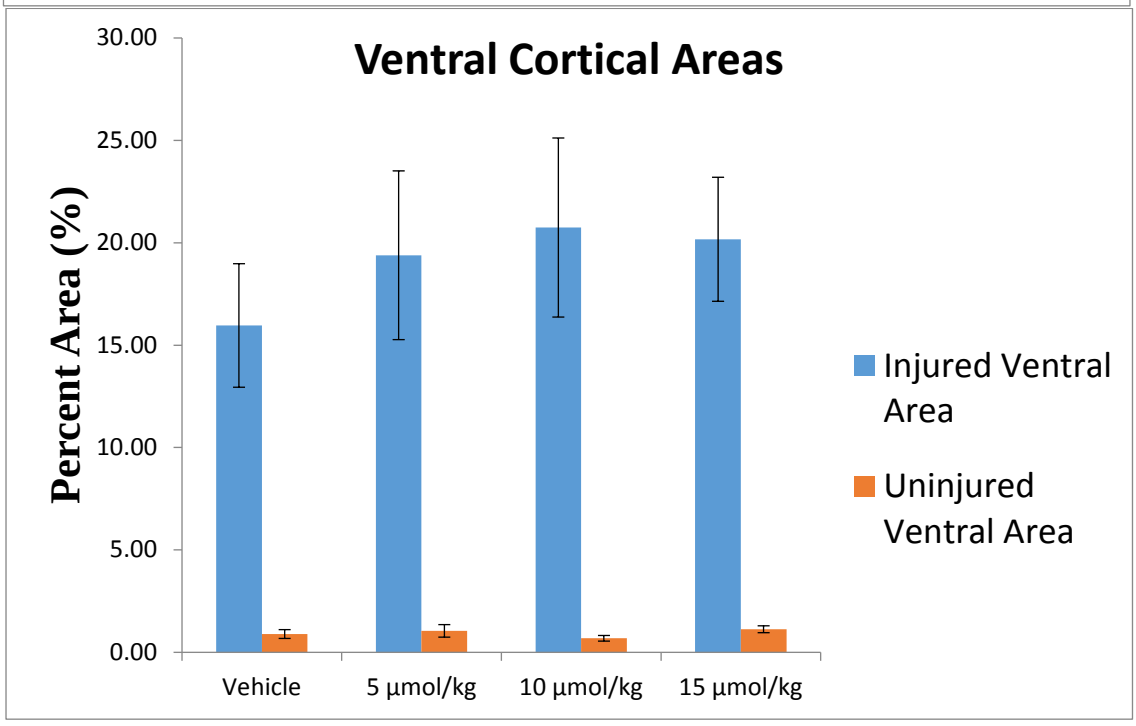


Figure 5
Comparisons between medial and ventral cortical areas. (5a) A significant difference between medial areas A and C were observed ($p < 0.001$) as expected, but no significant difference was observed between treatment groups. A non-significant trend, however, shows that the treatment groups endured higher microglial activation. (5b) A significant difference between ventral areas B and D were also observed ($p < 0.001$) as expected, but no significant difference was observed between treatment groups. Again, a non-significant trend was observed with the treated groups showing higher microglial activation than the vehicle.

DISCUSSION

The potential therapeutic use of connexin mimetic peptides to improve the outcome for individuals that suffer TBI was investigated in this study. No significant differences between the treatment groups was observed, contradicting the expected outcome. The connexin mimetic peptides, which theoretically blocked the gap junctions in the individual's brain, did not improve the outcome post-TBI. As expected, however, all individuals performed significantly worse post-TBI in behavioural testing regardless of treatment group. Uninjured hemispheres also showed significantly lower microglial activation using the Iba-1 analysis, though no significant differences was observed between treatment groups. It appeared that a non-significant trend of a worsening outcome for individuals receiving the connexin mimetic peptide treatment compared to the vehicle was observed. These results were consistent between behavioural, cellular, and immunohistochemical analyses.

Though few published studies have looked at the connection between gap junctions and TBI, experiments using connexin mimetic peptides have been conducted in spinal cord injury extensively. In one study regarding SCI, the treatment of connexin mimetic peptide for up to twenty-four hours post-SCI showed a decrease in microglial activation and improved hind limb function (O'Carroll, Gorrie et al. 2013). According to another study regarding the role of gap junctions in the spread of neuroinflammation, when the connexin43 gene is knocked out in a mouse model, the neuroinflammatory response is reduced post-SCI (Bennett, Garre et al. 2012).

Results from this study contradicted the findings in spinal cord injury. The current study did not demonstrate a neuroprotective effect from the use of connexin mimetic peptides. The non-significant trend suggests a potentially harmful effect of connexin mimetic peptide, which could be due to the time point at which the treatment was administered.

It is known that neuroinflammation occurs in two distinct stages: the acute and chronic stages. Initially, inflammation may prove to be beneficial for the injured brain (Morganti-Kossmann, Raghupathi et al. 2012). By administering the connexin mimetic peptide immediately post-TBI, the potentially beneficial and essential acute neuroinflammation may have been prevented, worsening the outcome for individuals receiving the peptide treatment. If this is the case, the administration of connexin mimetic peptides may prove to be a beneficial therapeutic treatment for people with TBI.

ACKNOWLEDGEMENTS

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