Neuroprotective Effect of Anthocyanin on Experimental Traumatic Spinal Cord Injury

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INTRODUCTION

Traumatic spinal cord injury (SCI) often leads to serious neurological sequelae and medical complications. The secondary damage following SCI is induced by multiple pathophysiological mechanisms, including vascular perturbation, metabolic failure, ionic dysregulation, and cellular excitotoxicity. These mechanisms increase blood-spinal cord barrier permeability, tissue edema, free radical formation, peroxidation of lipid membranes, cytokines release, and inflammation1,11,10. Among these mechanisms, the generation of reactive oxygen species (ROS), such as superoxide, H2O2, and hydroxyl radicals, is one of the main mechanisms in the acute injury of traumatic SCI14,26. Furthermore, oxidative injury due to ROS has complex interactions with excitotoxicity, apoptosis, and inflammation21. Therefore, antioxidants are hypothesized to be neuroprotective.

Anthocyanins are natural pigments belonging to the flavonoid family and are present in fruits and vegetables. It is known to have powerful antioxidant effects, inhibit inflammation, and have anticarcinogenic properties and potent cardioprotective effects13,19,28,33,34,17,41. Among anthocyanins, cyanidin 3-O-β-glucoside (C3G) has protective effects as a scavenger of active oxygen species in hepatic ischemia-reperfusion damage and cerebral ischemia models18,31,36. In addition, anthocyanins are taken up by brain endothelial cell lines and can possibly cross the monolayer in blood-brain barrier (BBB) in vitro model40. These results suggest that anthocyanins have potent protective effects in the oxidative stress-mediated disease of central nervous system (CNS). In 2006, two studies were published on pre-/post-treatment of C3G which showed significant improvement of neurological recovery and decrease of infarction volume in cerebral ischemia rat models18,31. However, the effects of anthocyanin have not yet been reported in traumatic SCI. In the present study, we aim to clarify whether treatment with anthocyanin (C3G) can reduce superoxide production, neuron cell damage, lesion volume, and neurological dysfunction in a rat model of traumatic SCI.

MATERIALS AND METHODS

Animal model of traumatic SCI

Forty adult male Sprague-Dawley rats weighing 250-300 g were
used. All procedures were performed in accordance with the guidelines for care and use of laboratory animals approved by Chung-Ang University’s Institutional Animal Care and Use Committee.

Moderate-grade SCIs were induced in adult rats using the pneumatic impact device (Chung-Ang University Hospital Model 2.0) as previously described. The contusion grade was controlled by depth of deformation, dwell time and velocity of impact. The initial BBB scores of moderate-grade SCI were 5 to 10. Anesthesia was induced with inhalation of 2.0% enflurane and total laminectomy of T10 was done with preservation of the dura. Because the diameter of impact tip was 2 mm, the exposed surface of the spinal cord had to be larger than 3 mm in diameter. After total laminectomy, the animal was placed in a prone position and its spine was fixed with spine clamps (Sang Chung Commercial Corporation, Seoul, Korea). The settings for the impact were 0.2 sec dwell time, 2 mm depth of deformation, and 3 m/sec velocity. The target point was the midline dorsal aspect of the spinal cord.

**Administration of anthocyanin**

The animals were divided into two groups: the vehicle-treated group (control group, n=20) which were received an oral administration of normal saline via stomach intubation immediately after SCI, and the anthocyanin-treated group (AT group, n=20) which were received 400 mg/kg of C3G in the same way. After the SCI, the wound was closed in layers using aseptic technique. Throughout the procedure, body temperature was maintained at 37°C with a heating pad. The bladder of the injured rats was manually emptied.

**Superoxide expression study**

To evaluate the antioxidant effects of anthocyanin during the acute stage of SCI, total twelve rats, six rats from each of the control and AT groups, were sacrificed at 1 hour after SCI by inhalation of 2.0% enflurane. The spinal cords were removed and frozen immediately in liquid nitrogen. The spinal cords were embedded in Tissue-Tek® O.C.T. Compound (Sakura Fine-tek, Zoeterwoude, Zoeterwoude, Netherland) and cut into 7 μm-thick axial sections. The sections were incubated with dihydroethidium (DHE) (5 mmol/L; Molecular Probes, Inc., Eugene, Ore., USA) in phosphate buffered saline (PBS) for 30 minutes at 37°C in a humidified chamber protected from light. DHE is oxidized by superoxide to ethidium bromide, which then binds to the DNA in the nucleus and fluoresces red. The red fluorescence was detected through a 543-nm long-pass filter, using a laser scanning microscope (LSM510 META; Carl Zeiss, Germany). Two regions of interest (ROIs) were selected at 1.5 mm (A, lesion periphery) and 3 mm (B, control) rostral from the epicenter (Fig. 1). The intensity of red fluorescence was measured using a VH image analyzer and the degrees of superoxide expression were represented by the ratio of red fluorescence in the two ROIs (A value divided by B value).

**Functional assessments**

All rats were examined in an open field environment to assess locomotor function of their hind limbs prior to injury, and at 1, 3, 5, 7, 10 and 14 days post injury using the BBB score, hind foot bar grab, and platform hang. All tests were performed by two investigators who were blinded to the study groups. The score for each animal was used as the average of the scores evaluating by the two investigators. When the two hind limb scores differed, the worse score was used for data analysis. Motor functions of the hind limbs were assessed by the BBB score. For example, a score of 0 (the lowest score) corresponds to no hind limb motion, while a score of 21 (the highest score) corresponds to normal motion. The hind foot bar grab test was used to test polysynaptic spinal reflexes. A score of 0 (the lowest score) was assigned if the rat did not respond to the bar touching with the hind feet, while a score of 3 (the highest score) was assigned if the rat strongly grabbed the bar and pushed it. The platform hang test was used to test the coordinated motor function of the hind limbs. A score of 0 (the lowest score) was assigned if the rat fell, while a score of 4 (the highest score) was assigned if the rat climbed the platform within 5 sec.

**Histopathological studies**

To assess histopathology, the remaining twenty-eight rats, fourteen rats from each of the control and AT groups, were sacrificed at 14 days after SCI. The rats were anesthetized by inhalation of 2.0% enflurane and transcardially perfused with 0.1 M PBS followed by 4% buffered paraformaldehyde. The spinal cords were then embedded in paraffin, cut into 5 μm-thick axial sections, and stained with hematoxylin and eosin (H&E) and Luxol...
fast blue (LFB) to identify motor neuron cell injury, lesion volume, and myelin loss. Motor neuron cells were counted in the anterior horns of 1.5 mm rostral from the epicenter. For each rat, the mean number of neuronal cells was obtained by examining at least three serial axial sections in the anterior horn. In each section, motor neurons were counted in three non-overlapping fields and were averaged into the cell number in HPF (×400) under light microscope. Only complete neuronal cells with a clearly defined cell body and nucleus were counted.

Lesion volume
The lesion volume was delineated by its bounding surface, which is defined by a series of closed contours in the H&E stained serial sections (Fig. 2). The software (OPTIMAS 6.5, Optimas, Inc., Bothel, WA, U.S.A.) measured the lesion volume from the two-dimensional images of the axial cord sections. The contours and structures in each spinal cord section were traced to reconstruct the stacked image for volumetric analysis. For each rat, the lesion area was computed using the section from the epicenter, 0.5, 1, 1.5, and 2 mm rostral and 0.5, 1, 1.5, and 2 mm caudal to the epicenter.

Statistical analysis
Data are expressed as means±standard deviation. Continuous data were compared using student t-test. Non-parametric data were compared using the Mann-Whitney U test. p values of <0.05 were considered statistically significant.

RESULTS

Superoxide concentration after SCI
Red fluorescence was higher at the lesion periphery (1.5 mm from the epicenter) at 1 hour after SCI in the control group, but was less prominent in the AT group (Fig. 3). The degrees of red fluorescence of DHE staining, which was calculated as ratios of the intensities at 1.5 (lesion periphery) and 3 mm (control) rostral from the epicenter, were 1.34±0.24 in the control group and 0.98±0.38 in the AT group, respectively. The mean value of the ratio was significantly lower in the AT group (p<0.05). This result indicates that treatment with anthocyanin significantly reduces superoxide concentration in the lesion periphery at 1 hour after SCI.

Functional recovery after SCI
The BBB score, platform hang and hind foot bar grab were used for the evaluation of functional outcomes (Table 1, Fig. 4). Before the injury, all rats showed normal function on the BBB score, platform hang and hind foot bar grab. At day 1 of SCI, the BBB scores of the control and the AT groups were 5.4±1.1 and 5.3±1.2, respectively, a difference that was not significant. However, they improved significantly at days 7, 10 and 14 after SCI (7 days : 11.7±0.9 and 13.8±1.0, 10 days : 12.6±0.8 and...
Lesion volumes in the control and the AT groups were 32.1±2.4 μL and 24.5±2.3 μL, respectively (Fig. 2). Anthocyanin significantly reduced the lesion volume by 23.7% in the AT group compared to that of the control group (p<0.05).

The motor neuron cell number were 8.3±5.1 cells/HPF in the control group and 13.4±6.3 cells/HPF in the AT group, respectively (Fig. 5) (p<0.05). The control group also showed more severe loss of myelin fibers compared with the AT group after SCI (Fig. 6).

**DISCUSSION**

Anthocyanins are present in fruits and vegetables, and are especially enriched in raspberries ("bok-bun-ja" in Korean). It provides natural pigmentation and exhibit a wide range of antioxidant and therapeutic benefits. Among the anthocyanins, C3G has known to have therapeutic effects in hepatic ischemia-reperfusion damage and cerebral ischemia models. In addition, it inhibits free radical-induced apoptosis of colon Caco-2 and myocardial cells, and enhances red blood cell resistance to oxidative stress. In these studies, the main therapeutic role of C3G is the scavenger of ROS. Furthermore, C3G was taken up by brain endothelial cell lines and could possibly cross the monolayer in blood-brain barrier in vitro model. From these results, we can speculate that C3G has potent protective effects in secondary injury of the traumatic SCI by ROS.

Superoxides are important in oxidative stress after traumatic SCI. The BBB score in the AT group only at days 7 and 14 were 11.7±0.9 and 13.8±1.0, significantly higher than the scores of 10.8±1.1 and 11.9±1.1 for the control group. In the AT group, the hind foot bar grab scores only at days 10 and 14 were 1.9±0.7 and 1.7±0.8, respectively. These scores were significantly higher than the scores of 1.3±0.6 (p<0.05) and 1.3±0.5 (p<0.05) for the control group.

**Table 1. Comparison of functional outcomes between the control and anthocyanin-treated groups after traumatic SCI**

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>Anthocyanin-treated group</th>
<th>p value</th>
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<tbody>
<tr>
<td>The BBB score</td>
<td></td>
<td></td>
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<tr>
<td>Pre-SCI</td>
<td>21</td>
<td>21</td>
<td>NS</td>
</tr>
<tr>
<td>Day 1</td>
<td>5.4±1.1</td>
<td>5.3±1.2</td>
<td>NS</td>
</tr>
<tr>
<td>Day 3</td>
<td>8.7±0.8</td>
<td>8.5±0.9</td>
<td>NS</td>
</tr>
<tr>
<td>Day 5</td>
<td>10.8±1.1</td>
<td>11.9±1.1</td>
<td>NS</td>
</tr>
<tr>
<td>Day 7</td>
<td>11.7±0.9</td>
<td>13.8±1.0</td>
<td>&lt;0.05</td>
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<tr>
<td>Day 10</td>
<td>12.6±0.8</td>
<td>14.7±0.9</td>
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<tr>
<td>Day 14</td>
<td>12.9±1.0</td>
<td>15.0±0.8</td>
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<tr>
<td>Platform hang</td>
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<td></td>
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</tr>
<tr>
<td>Pre-SCI</td>
<td>4</td>
<td>4</td>
<td>NS</td>
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<tr>
<td>Day 1</td>
<td>1.0±0.4</td>
<td>0.7±0.4</td>
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</tr>
<tr>
<td>Day 3</td>
<td>1.2±0.6</td>
<td>1.1±0.6</td>
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<tr>
<td>Day 7</td>
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<tr>
<td>Day 10</td>
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<td>1.3±0.7</td>
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<tr>
<td>Day 14</td>
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<td>1.4±0.9</td>
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<tr>
<td>Hind foot bar grab</td>
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<td></td>
</tr>
<tr>
<td>Pre-SCI</td>
<td>3</td>
<td>3</td>
<td>NS</td>
</tr>
<tr>
<td>Day 1</td>
<td>0.4±0.3</td>
<td>0.5±0.3</td>
<td>NS</td>
</tr>
<tr>
<td>Day 3</td>
<td>0.8±0.5</td>
<td>0.5±0.4</td>
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<tr>
<td>Day 5</td>
<td>0.8±0.6</td>
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<td>Day 14</td>
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<td>1.7±0.8</td>
<td>&lt;0.05</td>
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All data are presented as the means±standard deviations. SCI : spinal cord injury. BBB : blood-brain barrier, NS : not significant.

![Figure 4](image-url)
studies showed ranges of administration time and dosage of C3G, which showed a neuroprotective effect in CNS injuries. However, 11%, the difference of infarction volume between the two studies, was not higher than that of what we had expected. In 2000, Youdim and colleagues reported that of the oral ingest- ed 100 mg/kg C3G by rats (300 g), less than 0.64 μmol/L was found in the plasma within an hour following supplementa- tion\textsuperscript{39).} In contrast, Miyazawa and colleagues reported higher plasma levels of C3G by same rats (300 mg) even though their supplementation regimen, at approximately 45 mg and 90 mg, was less than that examined in Youdim’s study\textsuperscript{25).} These results suggest that differences in environment or condition of rats may have contributed to absorption potency. However, one parallel observation in various studies is that intact C3G rapidly appears in the plasma at 15 minutes after oral administration, with the maximal plasma concentration occurring 1 hour after oral administration of C3G via stomach intubation\textsuperscript{24,25,39).} These results could explain the reasonable outcome in Kang’s study despite low dosage and post-injury administration of C3G. In the present study, 400 mg/kg C3G was administrated via stomach intubation immediately after SCI. Considering SCI could influence the gastrointestinal motility and the rate of C3G absorption in rats, we used 400 mg/kg, higher dosage than previous studies\textsuperscript{14,31).} In the present study, the AT group showed significant improvement of the BBB score by 16.7% and the decrease of le-
sion volume by 23.7%, respectively. Those are not good outcomes compared to those of other free radical scavenger studies. Pretreatment of edaravone in SCI rat model improved the motor score by 27.3% and decreased the lesion volume by 36.4% at 7 days of SCI. Repeated treatment with Neu2000 resulted in a 45.6% decrease in overall lesion volume and approx. 34% improvement in the BBB score at 42 days of SCI. However, these absolute values could not imply the therapeutic inferiority of C3G, because the study design, such as administration time and method, contusion degree, and outcome measuring time, is different. Further studies with same conditions are needed to compare the therapeutic efficacy of various radical scavengers.

CONCLUSION

C3G has been shown to provide neuroprotective effects but its role in traumatic SCI was previously unexplored. The results of the present study demonstrate that C3G treatment significantly reduced the synthesis of ROS in the lesion periphery and improves functional outcomes associated with significant decreases in lesion volume and motor neuron injury. To best our knowledge, this study is the first report of C3G as a neuroprotective radical scavenger in traumatic SCI. However, we could not define the administration method, time, and pharmacokinetics for maximal effect, because this study reflected effects for single oral administration of 400 mg/kg C3G immediately after SCI. Further studies are necessary to evaluate the therapeutic time window, the appropriate administration method, and the proper dosage after trauma in order to establish the clinical usefulness of C3G for traumatic SCI.

Acknowledgements

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