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Jee Y. Lee, Hae Y. Choi, Tae Y. Yune

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Fluoxetine and vitamin C synergistically inhibits blood-spinal cord barrier disruption and improves functional recovery after spinal cord injury

Jee Y. Lee¹, Hae Y. Choi¹, and Tae Y. Yune¹, ²

¹Age-Related and Brain Diseases Research Center, ²Department of Biochemistry and Molecular Biology, School of Medicine, Kyung Hee University, Seoul, 02447, Korea

Correspondence should be addressed to:

Tae Y. Yune, Department of Biochemistry and Molecular Biology, School of Medicine, Kyung Hee University, Medical Building 10th Floor, 26, Kyungheedae-ro, Dongdaemun-gu, Seoul, 02447, Korea. Phone: +82-02-961-0968; Fax: +82-02-969-6343. E-mail address: tyune@khu.ac.kr
Abstract

Recently we reported that fluoxetine (10 mg/kg) improves functional recovery by attenuating blood spinal cord barrier (BSCB) disruption after spinal cord injury (SCI). Here we investigated whether a low-dose of fluoxetine (1 mg/kg) and vitamin C (100 mg/kg), separately not possessing any protective effect, prevents BSCB disruption and improves functional recovery when combined. After a moderate contusion injury at T9 in rat, a low-dose of fluoxetine and vitamin C, or the combination of both was administered intraperitoneally immediately after SCI and further treated once a day for 14 d. Co-treatment with fluoxetine and vitamin C significantly attenuated BSCB permeability at 1 d after SCI. When only fluoxetine or vitamin C was treated after injury, however, there was no effect on BSCB disruption. Co-treatment with fluoxetine and vitamin C also significantly inhibited the expression and activation of MMP-9 at 8 h and 1 d after injury, respectively, and the infiltration of neutrophils (at 1 d) and macrophages (at 5 d) and the expression of inflammatory mediators (at 2 h, 6 h, 8 h or 24 h after injury) were significantly inhibited by co-treatment with fluoxetine and vitamin C. Furthermore, the combination of
fluoxetine and vitamin C attenuated apoptotic cell death at 1 d and 5 d after injury and improved locomotor function at 5 weeks after SCI. These results demonstrate the synergistic effect combination of low-dose fluoxetine and vitamin C on BSCB disruption after SCI and furthermore support the effectiveness of the combination treatment regimen for the management of acute SCI.

Key words: blood spinal cord barrier, fluoxetine, vitamin C, matrix metalloprotease-9, inflammation

Introduction

Traumatic spinal cord injury (SCI) is a devastating condition that results in temporary or permanent loss of sensation, motor deficit or bowel/bladder dysfunction. Despite advances in understanding the pathophysiology of SCI, there is no cure at this time.

The blood spinal cord barrier (BSCB) is the functional equivalent of the blood-brain barrier (B-BB) in the sense of providing a specialized microenvironment for the cellular constituents of the spinal cord. When BSCB
is damaged by an injury, blood cells are infiltrated into the injured parenchyma and contribute to secondary injuries such as inflammation (Hawkins and Davis, 2005; Abbott et al., 2006; Zlokovic, 2008). These secondary injuries induce apoptotic cell death of neurons and glia, which results in permanent neurological deficits (Xu et al., 2001; Noble et al., 2002; Gerzanich et al., 2009). Emerging studies indicate that matrix metalloproteinase (MMPs) play critical roles in the BSCB disruption during acute SCI by degrading components of the extracellular matrix and tight junctions (TJ) in endothelial cells, leading to numerous pathological conditions including inflammation (Rosenberg et al., 1994; Rosenberg and Navratil, 1997; Noble et al., 2002; Lee et al., 2012b). Therefore, MMPs can be projected as a potential therapeutic target for treatment in SCI.

Fluoxetine, a selective serotonin reuptake inhibitor, is a FDA-approved drug widely used to treat depression, obsessive compulsive disorder, bulimia, and panic disorder (Mostert et al., 2008). Accumulating evidences also suggest that fluoxetine provides neuroprotective effects in various neurological disorders (Lim et al., 2009; Chung et al., 2011; Lee et al., 2012b; Scali et al., 2013; Lee et al., 2014). Especially, our recent studies showed that fluoxetine treatment
(immediately after injury and then daily injection for 14 d) significantly attenuates B-BB and BSCB disruption by inhibiting the expression and activation of MMP-9 and improves functional recovery in cerebral ischemia and SCI (Lee et al., 2012b; Lee et al., 2014).

Vitamin C, a potential antioxidant, is known to have protective effects via reducing or preventing oxidative damage (Santos et al., 2009; Iwata et al., 2014). Recently, it has been shown that high dose vitamin C treatment (2,000 mg/kg, daily for 14 d or 200 mg/kg, daily for 28 d) significantly improves functional recovery in rats with acute SCI (Robert et al., 2012; Yan et al., 2014). In addition, recent evidences showed that vitamin C prevents the disruption of B-BB and sustained the B-BB integrity in central nervous system (CNS) disease animal models such as sustained compression of primary somatosensory cortex, stroke, and Alzheimer’s disease (Lin et al., 2010; Kook et al., 2014; Allahtavakoli et al., 2015).

Here, we determined the effect of fluoxetine and vitamin C co-treatment (on BSCB breakdown and functional recovery after SCI. For the combination of both agents, we examined the dose level for each compound which did not show any effect of each drug administered alone. Consequently we selected a
low dose of fluoxetine (1 mg/kg) and vitamin C (100 mg/kg) and determined whether the combination treatment (daily for 14 d) exhibits synergistic effect on BSCB disruption, blood cell infiltration followed inflammation, apoptotic cell death, and neurological function after SCI.

**Materials & Methods**

**Spinal cord injury**

Adult rats [Sprague Dawley, male, 250-300 g; Sam:TacN (SD) BR; Samtako, Osan, Korea] were maintained under a constant temperature (23 ± 1 °C) and humidity (60 ± 10%) under a 12 h light/dark cycle (light on 07:30–19:30 h) with ad libitum access to drinking water and food. Prior to surgery, rats were weighed and anesthetized with chloral hydrate (500 mg/kg, intraperitoneal injection). An adequate level of anesthesia was determined by monitoring the corneal and hindlimb withdrawal reflexes. The back and neck regions were then shaved and laminectomy was performed at the T9-T10 level, exposing the cord beneath without disrupting the dura. The spinous processes of T8 and T11 were then clamped to stabilize the spine, and the exposed dorsal surface of the cord was subjected to moderate contusion injury (10 g x 25 mm) using a
New York University (NYU) impactor as described previously (Lee et al., 2010).

For the sham-operated controls, the animals underwent a T9-T10 laminectomy without weight-drop injury. After the injury the muscles and skin were closed in layers, and the rats were placed in a temperature and humidity-controlled chamber overnight. After spinal cord injury, the injured rats were randomly assigned to the placebo control or drug treated groups by a study assistant as a certain number of animals in each group. In this study, the following inclusion and exclusion criteria were adopted. The inclusion criteria were SD rats, weight between 250 and 300 g or general condition and normal motility. The exclusion criteria were: Death before breaking under anesthesia, out of injury range based on NYU impactor which calculates the parameters of trauma (eg. drop height, impact velocity, cord compression distance, cord compression rates) or normal movement in the first post-injury evaluation (21 points on the BBB scale of functional evaluation). Postoperatively, the rats were housed one per cage after injury with water and food easily accessible. Body weights and the remaining chow and water weight were recorded each morning for all animals. The bladder was emptied manually three times per day until reflexive bladder emptying was established. All surgical interventions and post-
operative animal care were performed in accordance with the Guidelines and Policies for Rodent Survival Surgery provided by the Animal Care Committee of the Kyung Hee University.

**Drug treatment**

Fluoxetine (Sigma, St. Louis, MO: 1, 5, or 10 mg/kg) and ascorbic acid (vitamin C, Sigma: 100, 200, 500, or 1,000 mg/kg) were dissolved in sterile PBS. Combination of fluoxetine (1 mg/kg) and vitamin C (100 mg/kg) was dissolved in sterile PBS and administered intraperitoneally (i.p) immediately after SCI and then further treated once a day for 14 d for analysis of behavioral analysis or for the indicated time points as previously described (Lee et al., 2012b). PBS was administered for vehicle control. For the sham-operated controls, animals underwent a T9-T10 laminectomy without contusion injury, and received no pharmacological treatment. All data obtained in this study were collected by the authors who were blinded to all treatment.

**Evans blue assay**

At 24 h after SCI, the permeability of the BSCB was investigated with Evans
Blue dye extravasation as previously described (Lee et al., 2012a). In brief, 5 ml of 2% Evans blue dye (Sigma) solution in saline was administered intraperitoneally. Three hours later, animals were anesthetized and sacrificed by intra-cardiac perfusion with saline. The T9 spinal cord segment was removed and homogenized in a 50% trichloroacetic acid solution. After homogenization, samples were centrifuged at 10,000 x g for 10 min, supernatants were collected and its fluorescence was quantified using a spectrophotometer at an excitation wavelength of 620 nm and an emission wavelength of 680 nm. Dye in samples was determined as micrograms per gram of tissue from a standard curve plotted using known amounts of dye.

**Tissue preparation**

At indicated time points after SCI, rats were anesthetized with chloral hydrate (500 mg/kg) and perfused via cardiac puncture initially with 0.1 M PBS and subsequently with 4% paraformaldehyde in 0.1 M PBS. A 20-mm section of the spinal cord, centered at the lesion site, was dissected out, post-fixed by immersion in the same fixative (4% paraformaldehyde) for 5 h and placed in 30% sucrose in 0.1 M PBS. The segment was embedded in OCT for frozen
sections, and serial transverse sections were then cut at 10 or 20 µm on a cryostat (CM1850; Leica, Germany). For molecular work, rats were perfused with 0.1 M PBS and segments of spinal cord (10 mm) including the lesion site were isolated and frozen at -80°C.

**RNA isolation and RT-PCR**

At 2 h, 6 h, 8 h and 24 h after injury, total RNA was isolated using TRIZOL Reagent (Invitrogen, Carlsbad, CA) and 0.5 µg of total RNA was reverse-transcribed into first strand cDNA using MMLV according to the manufacturer’s instructions (Invitrogen). The resulting cDNAs were subjected to real-time PCR with a Stratagene Mx3000P (Agilent Technologies, Waldbronn, Germany) using QuantiMix SYBR (PhileKorea Technology, Seoul, Korea) as described (Lee et al., 2012c). The primers were synthesized by the Genotech (Daejeon, Korea) and the primer sequences were summarized in Table 1.

**Western blot**

Segments of spinal cord (1 cm) including the lesion site were isolated at 4 h (for caspase-3), 1 d (for ZO-1, COX-2, iNOS), and 5 d (for caspase-3, ED-1) after
SCI. Total protein was prepared and Western blot analysis was performed with the lysates from spinal cord as previously described (Lee et al., 2012a). The primary antibodies used in Western blot analysis are as follows; cleaved caspase-3 (1:1,000, Cell Signaling Technology, Danvers, MA), ZO-1 (1:1,000, Invitrogen), occludin (1:1,000, Invitrogen), iNOS (1:10,000, Transduction Laboratory, Lexington, KY), COX-2 (1:1,000, Cayman Chemicals Ann Arbor, MI), and ED-1 (1:1,000, Serotec, Raleigh, NC). As loading controls, β-tubulin (1:10,000; Sigma) was used. The densitometric values of the bands on Western blots obtained by Alphalmager software (Alpha Innotech Corporation, San Leandro, CA) were subjected to statistical analysis. Background in films was subtracted from the optical density measurements.

**Gelatin zymography**

At 1 d after injury the activity of MMP-2 and MMP-9 was examined by gelatin zymography as previously described (Lee et al., 2012a). Relative intensity of zymography (relative to sham) was measured and analyzed by Alphalmager software (Alpha Innotech Corporation). Background was subtracted from the optical density measurements. Experiments were repeated three times and
the values obtained for the relative intensity were subjected to statistical analysis.

**Immunohistochemistry**

Frozen sections were processed for immunohistochemistry with antibodies against ZO-1 (1:100, Invitrogen), RECA-1 (Serotec), myeloperoxidase (MPO, 1:100, Carpinteria, CA) at 1 d and ED-1 (1:300, Serotec) at 5 d as previously described (Yune et al., 2007). For quantification of MPO- or ED-1-intensity, serial transverse sections (20 µm thickness) were collected every 100 µm rostral and caudal 3,000 µm to the lesion site (total 60 sections). Digital images of MPO- or ED-1-stained tissues were obtained and we quantified the fluorescent intensity above threshold by using MetaMorph software (Molecular devices, Sunnyvale, CA) and averaged. The threshold value was at least three times the background and the background was quantified and normalized to the primary antibody omitted control.

**TUNEL staining**

One and five days after injury, serial spinal cord sections (10 µm thickness)
were collected every 100 µm and processed for terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) staining using an Apoptag in situ kit (Millipore, Billerica, MA). Diaminobenzidine substrate kit (Vector Laboratories) was used for peroxidase staining, and the sections were then counterstained with methyl green. Control sections were incubated in the absence of TdT enzyme. Investigators who were blind as to the experimental conditions carried out all TUNEL analyses. For quantification, serial transverse sections (20 µm thickness) were collected every 100 µm section from 2 mm rostral to 2 mm caudal to the lesion epicenter (total 40 sections for neurons at 1 d) or 5 mm rostral to 5 mm caudal to the lesion epicenter (total 100 sections for oligodendrocytes at 5 d) as described (Lee et al., 2010). Only those cells showing morphological features of nuclear condensation and/or compartmentalization in the GM and WM were counted as TUNEL-positive. Some sections were processed for TUNEL and then for immunohistochemistry using specific cell type markers: NeuN (1:100, Millipore) for neurons; CC1 (1:100, Oncogene, Cambridge, MA) for oligodendrocytes. For double labeling, cyanine 3-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) was used and nuclei were labeled with
DAPI according to the protocol of the manufacturer (Invitrogen, Carlsbad, CA).

**Behavioral tests**

To test hindlimb locomotor function, open-field locomotion was evaluated at 1 d, 7 d, 14 d, 28 d, and 35 d after injury by using the Basso–Beattie–Bresnahan (BBB) locomotion scale as previously described (Basso et al., 1995; Yune et al., 2007). BBB is a 22-point scale (scores 0–21) that systematically and logically follows recovery of hindlimb function from a score of 0, indicative of no observed hindlimb movements, to a score of 21, representative of a normal ambulating rodent. BBB test was performed by trained investigators who were blind as to the experimental conditions. In this study, the rats that were used for behavioral studies were not used in the other assay.

**Assessment of lesion volume**

Lesion volume, using rats employed for behavioral analyses, was assessed as described previously (Yune et al., 2008). Serial longitudinal sections (10 μm) through the dorsoventral axis of the spinal cord were used to determine lesion volume. Every 50 μm, sections were stained with Cresyl violet acetate and
studied with light microscopy. With a low-power (1.25 X) objective, the lesion area was determined by MetaMorph software (Molecular devices). Areas at each longitudinal level were determined, and the total lesion volume derived by means of numerical integration of sequential areas.

**Statistical analysis**

Data presented as the mean ± SEM. Comparisons between two groups were made by unpaired Student's t-test. Multiple comparisons between groups were performed one-way ANOVA. Behavioral score was analyzed by repeated measures ANOVA (time vs. treatment). Tukey's multiple comparison was used as Post hoc analysis. Statistical significance was accepted with p<0.05. Statistical analyses were performed using SPSS 15.0 (SPSS Science, Chicago, IL).

**Results**

Co-treatment with vitamin C and fluoxetine synergistically inhibits BSCB disruption after SCI

Previously, we reported that a high dose of fluoxetine significantly inhibits the
increase of BSCB permeability in injured mouse spinal cord (Lee et al., 2012b). In addition, it has been shown that vitamin C prevents B-BB disruption in rat brain injury models (Lin et al., 2010; Allahtavakoli et al., 2015). To determine the effect of fluoxetine and vitamin C co-treatment on BSCB permeability after SCI, we assessed Evans blue assay at 1 d after injury. As shown in Fig. 1A, the amount of Evans blue dye extravasation increased after SCI when compared with the sham control, which indicates that SCI elicits BSCB disruption. Furthermore, fluoxetine (5 or 10 mg/kg) treatment after injury significantly reduced the amount of Evans blue dye extravasation when compared with the vehicle control. In addition, vitamin C (200, 500 or 1,000 mg/kg) treatment also inhibits Evans blue dye extravasation in dose dependent manner (Fig. 1B). However, a low dose of fluoxetine (1 mg/kg) or vitamin C (100 mg/kg) treatment only did not inhibit Evans blue dye extravasation (Fig. 1A, B).

Next, to determine the synergistic effect of fluoxetine and vitamin C co-treatment, we divided SCI-injured rats into four groups as follows: control group (PBS injection), vitamin C group (100 mg/kg of vitamin C only injection), fluoxetine group (1 mg/kg of fluoxetine only injection), and vitamin C+ fluoxetine
group [V+F, mixture of vitamin C (100 mg/kg) and fluoxetine (1 mg/kg) injection].

At this concentration of fluoxetine (1 mg/kg) and vitamin C (100 mg/kg), there was no inhibition effect on BSCB disruption after SCI (Fig. 1A, B). As shown in Fig. 1C and D, the Evans blue dye extravasation was not inhibited by only vitamin C or fluoxetine treatment, whereas co-treatment with vitamin C and fluoxetine (V+F) significantly inhibited BSCB permeability (Veh, 35.4 ± 1.9; Vit C only, 33.2 ± 2.6; Flu only, 31.6 ± 3.2; V+F, 18.6 ± 2.1, \( p < 0.05 \)). This result suggests the synergistic effect of vitamin C and fluoxetine co-treatment on BSCB disruption after SCI.

**MMP-9 expression and activation after SCI is inhibited by co-treatment with vitamin C and fluoxetine**

It has been known that the excessive proteolytic activity of MMPs such as MMP-2 and MMP-9 results in B-BB or BSCB disruption after CNS injury such as stroke and SCI (Rosenberg and Navratil, 1997; Rosenberg et al., 1998; Xu et al., 2001; Asahi et al., 2001; Noble et al., 2002). Because co-treatment with vitamin C and fluoxetine reduced BSCB disruption after SCI (Fig. 1), we examined whether co-treatment with vitamin C and fluoxetine would inhibit the expression
and activity of MMP-2 and/or MMP-9 after injury. As shown in Fig. 2A, the levels of MMP-2 and MMP-9 mRNA increased at 8 h after injury compared with the sham control. Furthermore, SCI-induced increase in Mmp-9 mRNA expression was significantly inhibited by co-treatment with vitamin C and fluoxetine compared with the vehicle control, whereas Mmp-2 mRNA expression was not (Fig. 2A, B). Using gelatin zymography, co-treatment with vitamin C and fluoxetine also significantly inhibited an increase in the activity of MMP-9 (active MMP-9 band) after SCI (Fig. 2C) as compared with the vehicle control (Fig. 2C, D) (active MMP-9; Veh, 8.7 ± 0.6 vs. V+F, 6.1 ± 0.7, p < 0.05).

**Co-treatment with vitamin C and fluoxetine prevents the degradation of tight junction proteins after SCI**

It is known that tight junctions (TJs) in the endothelial cells of the blood vessels maintain B-BB integrity (Zlokovic, 2008). The loss or degradation of TJ proteins has been implicated to mediate the hyperpermeability of BSCB after SCI (Lee et al., 2012a; 2012b). Thus, we next examined the effect of co-treatment with vitamin C and fluoxetine on the expression levels of the TJ proteins ZO-1 and occludin in the injured spinal lysates using Western blot
analysis. As shown in Fig. 3A and B, the decrease of the levels of ZO-1 at 1 d and occludin at 5 d after SCI as in previous report (Lee et al., 2012a) was significantly attenuated by co-treatment with vitamin C and fluoxetine as compared to vehicle control. In addition, double staining with ZO-1 and RECA-1 antibodies also showed that the fragmentation of capillary blood vessel was increased after SCI. However, co-treatment with vitamin C and fluoxetine significantly attenuated the capillary fragmentation (Fig. 3C), indicating that co-treatment with vitamin C and fluoxetine preserves TJ integrity by inhibiting loss of TJ proteins after injury.

Co-treatment with vitamin C and fluoxetine inhibits the infiltration of neutrophils and macrophages after SCI

Blood infiltration such as neutrophils and macrophages is followed by BSCB disruption and hemorrhage after SCI (Mun-Bryce and Rosenberg, 1998a;1998b). Since co-treatment with vitamin C and fluoxetine prevented BSCB disruption after SCI, we examined the effect of co-treatment with vitamin C and fluoxetine on the infiltration of blood cells by immunohistochemistry and Western blot analysis with the antibodies of MPO and ED-1. As shown in Fig.
4A, both MPO-positive neutrophils at 1 d and ED-1-positive macrophages at 5 d after injury were observed in the injured spinal cord. The relative fluorescence intensity analysis showed that co-treatment with vitamin C and fluoxetine significantly inhibited the infiltration of neutrophils and macrophages compared with the vehicle control (MPO: Veh, 1.0 ± 0.06 vs. V+F, 0.63 ± 0.09%, ED-1: Veh, 1.0 ± 0.08 vs. V+F, 0.52 ± 0.12, p < 0.05) (Fig. 4A, B). Western blot analysis also revealed that the increase of ED-1 level in the injured spinal cord lysates was significantly reduced by co-treatment with vitamin C and fluoxetine at 5 d after injury compared with the vehicle control (Fig. 4C, D). These findings suggest that the combination of a low dose of vitamin C and fluoxetine inhibits blood cell infiltration by preventing BSCB disruption after SCI.

It has also been known that both neutrophils and macrophages infiltrated after SCI produce inflammatory mediators such as IL-1β, TNF-α, COX-2 and iNOS and chemokines, such as Gro-α (CXCL-1), MCP-1 (CCL-2), MIP-1α (CCL-3), MIP-1β (CCL-4), and MIP-2α (CXCL-2), which thereby facilitate inflammatory responses (McTigue et al., 1998; Mun-Bryce and Rosenberg, 1998a; Ghirnikar et al., 2001; Ousman and David, 2001; Pineau and Lacroix, 2007). Thus, we next determined the effect of co-treatment with vitamin C and
fluoxetine on the expression of inflammatory mediators and chemokines after SCI. Using real-time RT-PCR, the increase of TNF-α, IL-1β (at 2 h), iNOS, and COX-2 (at 6 h) mRNA level after SCI were significantly inhibited by co-treatment with vitamin C and fluoxetine (Fig. 5A). The protein levels of iNOS and COX-2 at 1 d after injury were also significantly reduced by co-treatment with vitamin C and fluoxetine as compared with the vehicle control (Fig. 5B, C). In addition, co-treatment with vitamin C and fluoxetine significantly inhibited the increases in the mRNA levels of Gro-α, MIP-1α and MIP-1β at 8 h after injury (Fig. 5D).

Co-treatment with vitamin C and fluoxetine inhibits apoptotic cell death and improves functional recovery after SCI

It is known that infiltrating blood cells produce and release free radicals, nitric oxide, and MMPs, which directly cause tissue damage and apoptosis of neurons and oligodendrocytes after SCI (Hausmann, 2003). By TUNEL staining, we examined the effect of co-treatment with vitamin C and fluoxetine on apoptotic cell death in the GM at 1 d and in the WM at 5 d after SCI. Most TUNEL-positive cells in the GM at 1 d and in the WM at 5 d after SCI are neurons and oligodendrocytes respectively based on double-labeling (Fig. 6A').
as described (Lee et al., 2010). As a result, any positive signal was not observed in uninjured sham control (data not shown) and the co-treatment with vitamin C and fluoxetine significantly reduced the number of TUNEL-positive neurons and oligodendrocytes as compared to vehicle control (GM at 1 d: Veh, 323 ± 27; V+F, 203 ± 22; WM at 5 d: Veh, 205 ± 22; V+F, 122 ± 26, p ≤ 0.05) (Fig. 6B). Consistently, the increase of the cleaved (activated) forms of caspase-3 level at 4 h and 5 d after SCI was significantly alleviated by co-treatment with vitamin C and fluoxetine as compared to vehicle control (Fig. 6C, D). Therefore, our results indicate that the combination of vitamin C and fluoxetine may inhibit the apoptotic cell death of neurons and oligodendrocytes after SCI via the attenuation of BSCB disruption and blood cell infiltration.

To evaluate the effect of co-treatment with vitamin C and fluoxetine on functional recovery after SCI, rats were immediately treated with a mixture of vitamin C (100 mg/kg) and fluoxetine (1 mg/kg) and further treated once a day for 2 weeks after SCI. Functional recovery was then evaluated using the 21-point BBB scores (Basso et al., 1995) for locomotion. As a result, co-treatment with vitamin C and fluoxetine significantly increased the hindlimb locomotor function from 28 d to 35 d after injury as compared to that observed in vehicle-
treated control (At 35 d, Veh, 9.2 ± 0.7 vs. V+F 11.5 ± 0.8, \( p < 0.05 \)) (Fig. 6E).

When tissue loss with serial longitudinal sections after Cresyl violet staining was evaluated, the total lesion volume was significantly decreased upon co-treatment with vitamin C and fluoxetine as compared with that of vehicle treatment (Veh, 10.2 ± 1.3; V+F, 5.4 ± 0.9 mm\(^3\), \( p \leq 0.05 \)) (Fig. 6F, G).

**Discussion**

In the present study, we for the first time demonstrated that a low-dose combination of fluoxetine (1 mg/kg) and vitamin C (100 mg/kg) synergistically exerts neuroprotective effects by inhibiting MMP-9 activation and thereby preventing BSCB disruption after SCI. Co-treatment with fluoxetine and vitamin C also reduced the number of infiltrating blood cells such as neutrophils and macrophage and inhibited pro-inflammatory cytokine and chemokines, thereby resulting in reduced inflammatory responses. In addition, apoptotic cell death of neurons and oligodendrocytes was inhibited and functional recovery was improved after SCI by post-treatment of the combination of fluoxetine and vitamin C. Thus, our findings provide a possibility that the combination therapy with fluoxetine and vitamin C can be effective in
neurological diseases accompanying B-BB or BSCB disruption such as traumatic or ischemic brain injury including SCI.

It has been known that a high dose of fluoxetine (10 mg/kg) or vitamin C (500 mg/kg) inhibits B-BB or BSCB disruption after brain ischemia and SCI (Lee et al., 2012b; Lee et al., 2014). In addition, vitamin C (500 mg/kg) treatment prevented B-BB disruption in rat brain injury models (Lin et al., 2010; Allahtavakoli et al., 2015). Furthermore, a high-dose of vitamin C supplementation (33 g/l) was reported to reduce amyloid plaque burden and ameliorate pathological change in the brain of 5XFAD mice (Kook et al., 2014).

As shown in fig. 1A and B, BSCB disruption after SCI was not inhibited by a low dose of fluoxetine (1 mg/kg) or vitamin C (100 mg/kg) alone treatment. However, we found that co-treatment with fluoxetine and vitamin C significantly reduced the extravasation of Evan blue dye after SCI, indicating the synergistic effect of co-treatment with vitamin C and fluoxetine on BSCB disruption. It has also been known that B-BB or BSCB disruption under various pathological conditions such as stroke and SCI results in increased cerebrovascular permeability with subsequent development of tissue edema (Utepbergenov et al., 1998). Especially, the upregulation of MMP-9 has been implicated in
BSCB disruption followed SCI-induced secondary damage by degrading the basal components of BSCB and facilitating immune cell infiltration (Noble et al., 2002), although many factors are known to contribute to B-BB or BSCB disruption. Our data also showed that co-treatment with fluoxetine and vitamin C significantly inhibited MMP-9 expression and activity after SCI (see Fig. 2). Additionally, blood infiltration and loss of TJ proteins were significantly attenuated by co-treatment with vitamin C and fluoxetine. These results suggest that the attenuation of BSCB disruption by co-treatment with vitamin C and fluoxetine is in part mediated by inhibiting MMP-9 expression and activation after SCI.

Reactive oxygen species (ROS) as well as reactive nitrogen species such as nitric oxide and peroxynitrite have been known to play an important role in the B-BB disruption and endothelial cell permeability (Parathath et al., 2006; Schreibelt et al., 2007). Additionally, superoxide anion, one of the major ROS has been known to regulate the endothelial TJ proteins and increase the B-BB permeability through RhoA, phosphatidylinositols 3 kinase, and the protein kinase B (PKB/AKT) signaling pathway (Sheth et al., 2003; Schreibelt et al., 2007). The report by Lin et al. (2010) also showed that B-BB disruption
induced by transient compression of somatosensory cortex was ameliorated by vitamin C treatment (500 mg/kg) by inhibiting ROS-mediated oxidative stress. Therefore, we can’t exclude a possibility that the inhibitory effect by the combination of a low dose of fluoxetine and vitamin C on BSCB disruption and functional recovery after SCI may be mediated by anti-oxidant effect of vitamin C and further study about this view is needed.

Blood vessels including capillaries are ruptured immediately by mechanical injury itself and further fragmented by various factors such as metalloproteases and sulfonylurea receptor 1 induced after SCI (Simard et al., 2007; Simard et al., 2010; Zhang et al., 2011), thereby, blood infiltration is increased further. In this study, we found that the fragmentation of blood vessels was increased after SCI as previously reported (Simard et al., 2010). Furthermore, co-treatment with vitamin C and fluoxetine significantly inhibited the fragmentation of capillaries as compared with vehicle control (see Fig. 3B). However, SCI-induced sulfonylurea receptor 1 expression was not inhibited by co-treatment with vitamin C and fluoxetine (data not shown), indicating the inhibition of the fragmentation of capillary blood vessels by co-treatment with vitamin C and
fluoxetine after SCI was mediated in part by attenuating MMP-9 expression and activation.

It has been known that B-BB or BSCB disruption followed infiltration of blood cells including macrophages also results in tissue damage after SCI and the inhibition of blood cells infiltration ameliorates apoptotic cell death and improves functional recovery (Hamada et al., 1996; Taoka et al., 1997; Saiwai et al., 2010). Co-treatment with vitamin C and fluoxetine also significantly attenuated apoptotic cell death (see Fig. 6A-D), and improved functional recovery after SCI (see Fig. 6E). Thus, our data suggest that the neuroprotective effect of co-treatment with vitamin C and fluoxetine might be mediated in part by preventing BSCB disruption followed blood cells infiltration after SCI. Oxidative stress by ROS produced after SCI also is known to play an important role in apoptotic cell death. The report by Chen et al. (Chen et al., 2014) showed that the combined administration of vitamin C (240 mg/kg) and E (200 mg/kg) prevents programmed cell death after SCI through an anti-oxidant effect. In addition, it was known that a high dose of vitamin C (200 or 2,000 mg/kg) improves functional recovery after SCI in rats (Liao et al., 2001; Robert et al., 2012; Yan et al., 2014). Thus, there is a possibility that vitamin C as an anti-oxidant can
protect apoptotic cell death after SCI, although the concentration of vitamin C (100 mg/kg) used in this study was low. However, it should be pointed out that the precise action mechanism of how low dose of vitamin C potentiate the effect of low dose of fluoxetine is still unknown.

Collectively, the present study indicated that co-treatment with a low-dose vitamin C and fluoxetine exhibits the synergistic neuroprotective effect after SCI, which is mediated by the inhibition of MMP-9 expression and activation followed BSCB disruption, thereby attenuating inflammatory response. Considering that fluoxetine is currently used clinically as an antidepressant and vitamin C has no cytotoxic effect in human, our results suggest that the combination therapy with vitamin and fluoxetine may provide a potential therapeutic intervention for preventing BSCB or B-BB disruption after SCI and ischemic injury.

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Figure legends

Table 1. Primer sequences used for real time RT-PCR

Figure 1. Co-treatment with vitamin C and fluoxetine synergistically inhibits BSCB disruption after SCI. Vehicle (PBS), fluoxetine (1, 5, 10 mg/kg), vitamin C (100, 200, 500, or 1,000 mg/kg), and mixture of vitamin C (100 mg/kg) and fluoxetine (1 mg/kg) were administered by i.p. injection immediately after injury, and rats were sacrificed at 24 h (1 d) and processed for Evans blue assay as described in the Methods sections (n = 5). (A) Quantification of the Evans blue
extravasation by using fluorometer (excitation at 620 nm and emission at 680 nm). Note that Evans blue extravasation was significantly reduced in 5 and 10 mg/kg of fluoxetine-treated group as compared with vehicle control. (B) Quantification of the Evans blue extravasation. Note that Evans blue extravasation was significantly reduced in 200, 500, and 1,000 mg/kg of vitamin C-treated group as compared with vehicle control. (C) Representative whole spinal cords demonstrating that Evans blue dye was permeabilized into spinal cord at 1 d after injury. (D) Quantification of the Evans blue extravasation. Note that Evans blue extravasation was significantly reduced in a mixture of vitamin C (100 mg/kg) and fluoxetine (1 mg/kg)-treated rats (V+F) compared with vehicle-treated rats (Veh), whereas 100 mg/kg of vitamin C (Vit) and 1 mg/kg of fluoxetine (Flu)-treated rats was not reduced Evans blue extravasation. The value is presented as amount of dye (µg)/tissue weight (g). Data are presented as the mean ± SEM. *p < 0.05 vs. Veh.

Figure 2. Co-treatment with vitamin C and fluoxetine inhibits the expression and activation of MMP-9 after SCI. (A) RT-PCR for Mmp-2 and Mmp-9 at 8 h after SCI. (B) Densitometric analyses for Mmp-2 and Mmp-9 (n = 3). (C) Gelatin
zymography at 1 d after SCI. (D) Densitometric analysis for zymography (n = 4). Note that V+F treatment significantly inhibited MMP-9 expression and activation after SCI. Data are presented as the mean ± SEM. *p < 0.05 vs. Veh.

**Figure 3.** Co-treatment with vitamin C and fluoxetine prevents the degradation of tight junction proteins after SCI. To examine the degradation of tight junction proteins after SCI, vehicle or V+F were injected into the injured rats and total protein was extracted from the spinal cord at 1 d and 5 d after injury (n = 3/group). (A) Western blots for ZO-1 (at 1 d after SCI) and occludin (at 5 d after SCI). (B) Quantitative analysis of the Western blots. The data are presented as the mean ± SEM. The levels of significance was *p ≤ 0.05 vs Veh. (C) Representative micrographs showing double immunofluorescence with ZO-1 and RECA-1 (endothelial cell marker) at 500 µm caudal to the lesion epicenter. Scale bar, 50 µm.

**Figure-4.** Co-treatment with vitamin C and fluoxetine attenuates the infiltration of neutrophils and macrophages after SCI. After SCI, V+F were treated and
blood cell infiltration was assessed as described in the Methods section. (A) Representative photographs of MPO-labeled neutrophils or ED-1-labeled macrophages in the dorsal column of injured spinal tissues treated with or without V+F are shown. The representative sections were collected 2 mm rostral to the lesion epicenter. Scale bar, 100 µm. (B) Quantitative analysis of immunoreactivity of MPO- or ED-1-positive cells (n = 5). (C, D) Western blot and densitometric analysis for ED-1 at 5 d after injury (n = 3). Note that V+F treatment significantly inhibited the infiltration of blood cells after SCI when compared with vehicle control. Data are presented as the mean ± SEM. *p < 0.05 vs. Veh.

**Figure-5.** Co-treatment with vitamin C and fluoxetine inhibits the expression of inflammatory factors and chemokines after SCI. Total RNA and protein extracts from vehicle or V+F-treated spinal cords were prepared at the indicated time points after injury. (A) real-time RT-PCR of *IL-1β*, *Tnf-α* (at 2 h), *Cox-2*, and *iNos* (at 6 h) after injury (n = 3). (B, C) Western blots (B) and quantitative analysis (C) of *iNos* and *Cos-2* at 1 d after injury (n = 3). (D) real-time RT-PCR of *Gro-α*, *Mip-2α*, *Mcp-1*, *Mip-1α*, and *Mip-1β* (at 1 d) mRNA expression after
injury (n = 3). Data are presented as the mean ± SEM. *p < 0.05 vs. Veh.

**Figure-6.** Co-treatment with vitamin C and fluoxetine inhibits apoptotic cell death and improves functional recovery after SCI. After SCI, rats were treated with vehicle or V+F and spinal tissues and extract were prepared for TUNEL staining and Western blot. (A) Representative image of TUNEL staining at 1 d (GM) and 5 d (WM) after injury. (A’, upper panels) TUNEL-positive neurons (arrows) in the GM at 1 d after injury. (A’, bottom panels) TUNEL-positive oligodendrocytes (arrows) in the WM at 5 d after SCI. Representative images were from the sections selected 1 mm (1 d, GM) or 5 mm (5 d, WM) rostral to the lesion epicenter. Scale Bars, 30 µm. (B) Quantitative analysis of TUNEL-positive cells (n = 5). (C) Western blots of cleaved caspase-3 at 4h and 5 d after SCI. (D) Quantitative analyses of Western blots (n = 3). (E) Functional recovery in injured rats was assessed with BBB locomotor score (n = 15). (F) Representative spinal cord tissues (1.2 mm from the dorsal surface) showing cavitation in the lesion site at 38 d after injury. (G) Quantitative analysis of the lesion area (n = 5). Note that co-treatment of vitamin C and fluoxetine significantly decreased the lesion volume at 38 d after injury. The data are
presented as the mean ± SEM. The levels of significance was *$p \leq 0.05$ vs. Veh.
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Highlights

- Combination of low-dose fluoxetine and vitamin C prevents BSCB disruption after SCI.
- Combination of low-dose fluoxetine and vitamin C inhibits apoptosis after SCI.
- Combination of low-dose fluoxetine and vitamin improves functional recovery after SCI.