

ORIGINAL ARTICLE

Effects of the combined administration of vitamins C and E on the oxidative stress status and programmed cell death pathways after experimental spinal cord injury

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Study Design: Experimental, controlled, animal study.

Objectives: To assess the effects of vitamins C and E (VCE) treatment on oxidative stress and programmed cell deaths after rat spinal cord injury (SCI), as well as functional recovery.

Setting: Taiwan.

Methods: Fifty-four Sprague–Dawley rats were used for the experimental procedure. In the sham group, laminectomy at T10 was performed, followed by impactor contusion of the spinal cord. In the control group, only a laminectomy was performed without contusion. Oxidative stress status was assessed by measuring the spinal cord tissue content of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities. We also evaluated the effects of combined VCE treatment using western blot to analyze expression of cleaved caspase-3 and microtubule-associated protein light chain 3 (LC3), and the Basso, Beattie and Bresnahan (BBB) scale to evaluate functional outcomes.

Results: Combined treatment of VCE significantly counteracted the effects of spinal cord contusion on oxidative stress represented by activities of SOD and GSH-Px ($P < 0.05$). The VCE treatment also significantly enhanced LC3-II expression and decreased cleaved caspase-3 compared with the sham ($P < 0.05$). Furthermore, BBB scores significantly improved in the VCE-treated group compared with the sham group (on day 14 and 28 after SCI; $P < 0.05$).

Conclusions: The combined administration of VCE was clearly capable of modulating the antioxidant effects, and of reducing apoptosis and increasing autophagy at the lesion epicenter leading to an improved functional outcome. Use of such clinically ready drugs could help earlier clinical trials in selected cases of human SCIs.

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Keywords: Vitamin C; Vitamin E; oxidative stress; programmed cell death; cord injury

INTRODUCTION

The pathologic course of spinal cord contusion injuries includes primary and secondary mechanisms of injury. Oxygen free radicals and lipid peroxidation were suggested as important factors in secondary injury to the spinal cord. After a primary mechanical injury has occurred, some secondary injury may respond to pharmacologic interventions. However, only limited therapeutic measures are currently available for treating human spinal cord injuries (SCIs). Despite many clinical trials having been carried out, the only treatment to date known to ameliorate neurological dysfunction is intravenous methylprednisolone therapy, and even it is being challenged in recent years.¹

The spinal cord, a part of the central nervous system, mostly comprises lipids and is easily destroyed by free radicals and the lipid peroxidation they induce. Hence, free radical-induced lipid peroxidation is believed to be important in the secondary destruction of damaged spinal cords.² Antioxidants have been evaluated as neuroprotective agents in stroke, as there is evidence supporting the occurrence of oxidative stress in the ischemic brain.³ Pharmacological studies in animals showed that antioxidant molecules are able to

cross the blood–brain barrier, such as polyethylene glycol-conjugated superoxide dismutase (SOD) and catalase, to reduce ischemic cerebral damage.⁴

Vitamins C and E (VCE) are antioxidants that are thought to have protective effects by reducing or preventing oxidative damage. Water-soluble vitamin C is found in the cytosol and extracellular fluid and can directly interact with free radicals to prevent oxidative damage. Vitamin E is a lipid-soluble antioxidant, which prevents lipid peroxidation chain reactions in cellular membranes by interfering with the propagation of lipid radicals. Most of the effects of the clinically used methylprednisolone are believed to be derived from its antioxidant capacity. It scavenges lipid peroxides in cell membranes and alleviates damage mediated by oxygen-derived free radicals.⁵ Hence, due to controversies surrounding the use of methylprednisolone clinically, we introduced combined treatment with VCE to assess the protective effects of these antioxidants.

Our previous research showed the existence of autophagy after SCIs in rats and the feasibility of manipulating the expression of autophagy to improve functional outcomes.^{6,7} The protective effects of VCE against apoptotic cell death are well described in the literature.^{8,9}

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Some studies also demonstrated associations of VCE with the expression of autophagy.^{10,11} In this study, we hypothesized that antioxidant therapy using combined treatment with VCE may protect the injured cord from deleterious effects of oxidative stress after an SCI, influence SCI-induced programmed cell death and thereby promote locomotor recovery after an SCI.

MATERIALS AND METHODS

Animal preparations and SCI

Adult female Sprague–Dawley rats (BioLASCO, Ilan, Taiwan) weighing 260–320 g were used for the experiments. Animals were maintained for at least 7 days before the experiment in a temperature-regulated room (23–25 °C) on a 12-h light/dark cycle. Food was withheld, but rats were allowed free access to water overnight before the surgery. All experimental protocols were in compliance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of the Chang Gung Memorial Hospital (Keelung, Taiwan).

The method for inducing SCI was developed and adapted by the Multicenter Animal Spinal Cord Injury Study, as described in detail by Gruner¹² and used a Multicenter Animal Spinal Cord Injury Study impactor (W.M. Keck Center for Collaborative Neuroscience, Piscataway, NJ, USA). Briefly, rats were anesthetized with an intraperitoneal injection of Rompun (xylazine at 10 mg kg⁻¹ body mass; Bayer, Leverkusen, Germany) and Zoletil (tiletamine and zolazepam at 40 mg kg⁻¹ body mass; Virbac, Carros, France). A laminectomy was performed at the caudal portion of T9 and all of T10 to expose the underlying cord without disrupting the dura. The exposed dorsal surface of the cord was subjected to the impact of a dropped weight using a 10 g rod released from a height of 12.5 mm, which is consistent with moderate severity of SCI. The lesion severity was verified by the impact velocity of the impact rod immediately after injury. Animals with an impact velocity error exceeding 5% were excluded from further study. After the SCI, rats were placed in individual cages and underwent manual bladder expression three times per day until reflexive bladder emptying was established. Control group animals received the same surgical procedures, but impaction was not applied to the spinal cord.

Drug administration

To examine the influence of VCE treatment after an SCI, vitamin E ((±)-α-tocopherol; Sigma T-3251, St Louis, MO, USA) was intraperitoneally administered at a dose of 200 mg kg⁻¹ body mass, and vitamin C (sodium ascorbic; Sintong Vitacicol, Taiwan) was intraperitoneally injected at a dose of 240 mg kg⁻¹ body mass in the treatment group once daily for 7 days. The VCE treatment dose and frequency were modified from our previous studies^{6,7} and reviewed literatures.^{13,14}

Western blot analysis

On the seventh day after the SCI, the control, sham-operated and treated ($n=6$) rats were anesthetized and underwent intracardiac perfusion with 0.1 mol l⁻¹ phosphate-buffered saline (pH 7.4). Proteins were prepared from spinal cord tissue obtained from the lesion epicenter (2.5 mm cephalad and caudal), and the protein concentration was determined using a BCA protein assay kit (Pierce Chemical, Rockford, IL, USA). Proteins (30 µg) were separated on a 4–20% gradient polyacrylamide gel (BioRad, Hercules, CA, USA) and transferred onto nitrocellulose membranes. Membranes were incubated with a monoclonal antibody directed against α-cleaved caspase-3 (1:500; Cell Signaling Technology, Danvers, MA, USA), rabbit polyclonal antibody microtubule-associated protein light chain 3 (LC3) (1:200; Cell Signaling Technology), and subsequently with a secondary antibody linked to horseradish peroxidase (1:5000; Sigma). The bands were visualized by using the VersaDoc 4000 Imaging System (BioRad). Relative densities of the bands were analyzed with Quantity One (vers. 4.5.2, BioRad). Band densities were normalized using β-actin.

Measurement of SOD and GSH-Px activity levels

The oxidative stress status was assessed by measuring the spinal cord tissue activities of SOD (#574601) and glutathione peroxidase (GSH-Px) (#353919; EMD/Calbiochem, San Diego, CA, USA). SOD and GSH-Px activities are respectively expressed in units per gram and nanomole per milligram of spinal cord tissue. SOD activity was detected with the Calbiochem Superoxide Dismutase Assay Kit II, which utilizes a tetrazolium salt for detecting superoxide radicals generated by xanthine oxidase and hypoxanthine, and samples were spectrophotometrically measured at 420 nm. The SOD assay measures all three types of SOD (Cu/Zn-, Mn- and Fe-SOD). GSH-Px activity is coupled with the oxidation of reduced nicotinamide adenine dinucleotide phosphate by glutathione reductase. The oxidation of nicotinamide adenine dinucleotide phosphate was followed spectrophotometrically at 340 nm using an enzyme-linked immunosorbent assay plate reader at 37 °C. The assay was performed with minor modifications to the manufacturer's directions.

Functional assessment

All outcome measures were obtained in a blinded fashion by two investigators and averaged. The score was assessed before injury and at 1, 4, 7, 10, 14, 21 and 28 days postoperatively (d.p.o.), by open-field testing using the methodology of Basso, Beattie and Bresnahan.¹⁵

Statistical analysis

All data were analyzed using SPSS statistical software (Chicago, IL, USA). Data are expressed as the mean ± s.e.m. Multiple group comparisons of differences in quantitative measurements were made by Kruskal–Wallis H nonparametric tests. For the BBB score data, we used an analysis of variance with repeated measurements over time. We determined the significance of any differences in the BBB scores at each time point by a nonparametric Kruskal–Wallis test. In all analyses, we considered a *P*-value of <0.05 to be statistically significant.

We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during the course of this research.

RESULTS

Effects of VCE on SOD and GSH-Px levels in cord tissues of SCI rats

To verify the antioxidant effects after VCE treatment, we measured the activities of two biomarkers of oxidative stress (SOD and GSH-Px) 7 days after injury. SOD and GSH-Px activities of each group were compared ($n=6$). We found that SOD had decreased in rats at 7 days after SCI, whereas VCE administration increased levels of SOD in SCI rats, compared with untreated SCI rats (*P*<0.05; Figure 1a). We also measured GSH-Px levels and found that SCI resulted in elevated GSH-Px levels in rats 7 days after SCI. VCE treatment reduced levels of GSH-Px in SCI rats compared with those in untreated SCI rats (*P*<0.05; Figure 1b).

Effects of VCE on programmed cell death derived from an SCI

We next examined the influence of combined vitamin treatment on different programmed cell death pathways. In order to quantify the amount of apoptosis expressed after the SCI relative to the control, we examined the level of cleaved (activated) caspase-3 in spinal cord tissue 7 days after injury ($n=6$). We found that the expression of cleaved caspase-3 increased after spinal cord contusion. In contrast, VCE treatment reduced the elevated levels of cleaved caspase-3 in rats with an SCI (Figure 2a). Densitometric quantification of protein band densities demonstrated a significant decrease (*P*<0.05) in the level of cleaved caspase-3 in treated vs sham-operated animals (Figure 2b). To evaluate the effect of VCE treatment on the expression of autophagy after an SCI, we examined levels of LC3 II expression at 7 d.p.o. using a western blot analysis (Figure 2a). Microtubule-associated protein

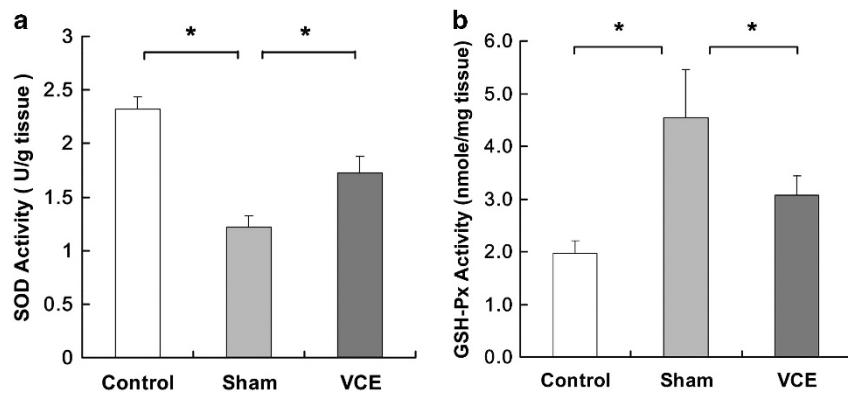


Figure 1 Effects of VCE treatment on levels of SOD and eGSH-Px in rats with a SCI. (a) Treatment with VCE reversed levels of SOD in SCI rats compared with the sham-operated group. (b) Treatment with VCE also reversed levels of GSH-Px in SCI rats. Levels of SOD and GSH-Px were detected by an assay kit and measured by spectrophotometry. * $P<0.05$.

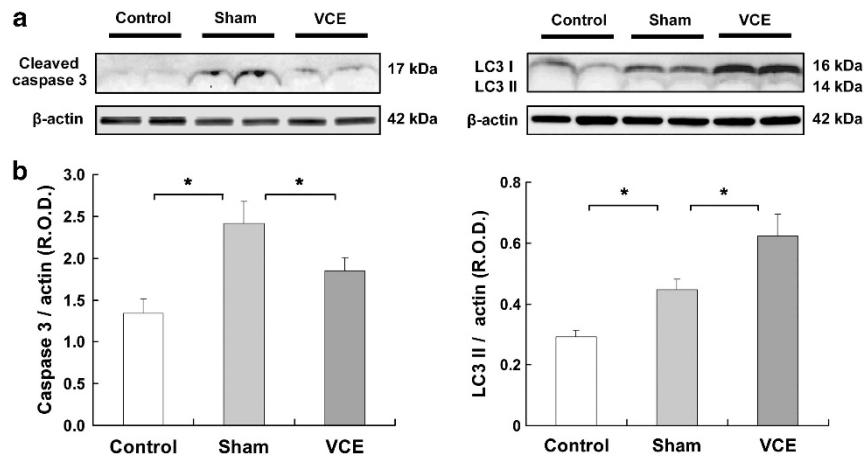


Figure 2 (a) Western blot analysis of cleaved caspase-3 and LC3-II expression in spinal cords of control, sham-operated, and VCE-treated rats. We used β -actin as a loading control (samples from two and four of six rats are shown). (b) Left: Quantitative analysis of cleaved caspase-3 protein levels illustrated by cleaved caspase-3/ β -actin (relative optical density; ROD). Acute intraperitoneal administration of VCE after injury significantly reduced cleaved caspase-3 levels. Right: Quantitative analysis of LC3-II protein levels illustrated by LC3-II/ β -actin (ROD). LC3-II expression was upregulated in VCE-treated spinal cords. Data are expressed as the mean \pm s.e.m. ($n=6$ per group, * $P<0.05$; nonparametric Kruskal-Wallis test).

LC3, a mammalian homolog of yeast *Atg8*, is an essential marker for detecting the expression of autophagy. In contrast, we found LC3 II expression to be reduced after an SCI, and VCE treatment significantly enhanced LC3 II expression (Figure 2b).

Effects of VCE on locomotor functional recovery in rats with an SCI

To evaluate the effect of VCE treatment on locomotor recovery after an SCI, BBB scores were measured for 4 weeks (Figure 3) ($n=6$). BBB scores of VCE-treated and vehicle-treated (sham-operated) rats had increased after 4 days and had reached a plateau at 14 days after injury. Average BBB scores were consistently higher in VCE-treated rats than in vehicle-treated rats at 4–28 days after injury. At 14 and 28 days after injury, animals that underwent VCE treatment (BBB scores of 10.5 ± 0.87 at 14 d.p.o. and 13.25 ± 0.75 at d.p.o. 28) performed better than SCI animals (BBB scores of 8.5 ± 0.29 at 14 d.p.o. and 10.5 ± 0.54 at 28 d.p.o.), and the difference was statistically significant ($P<0.05$).

DISCUSSION

SCI causes tissue damage through both primary and secondary injuries. Lipid peroxidation and apoptosis (that is, type II programmed cell death) are believed to be two of the most important factors precipitating posttraumatic degeneration due to secondary destruction of damaged spinal cords. Our current results show that combined VCE administration significantly normalized levels of oxidative stress and counteracted apoptotic cell death in animals that had experienced an SCI. In addition, the combined treatment significantly enhanced the expression of autophagy, which is reported to be important for maintaining synaptic plasticity.¹⁶ In addition, combined treatment with VCE produced significantly improved locomotor outcomes in rats after an SCI. These results suggest that multifaceted effects of combined VCE treatment may promote resistance to some secondary injuries after an SCI.

VCE treatment modulates oxidative stress

Spinal cord tissues are highly vulnerable to oxidative stress because of the abundance of polyunsaturated fatty acids in the spinal cord that

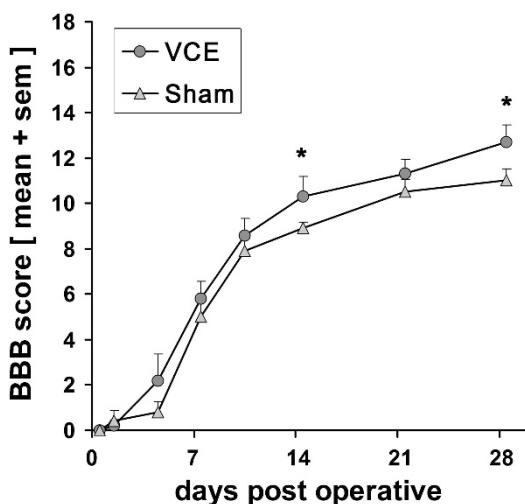


Figure 3 Behavioral analyses of rats receiving a spinal cord contusion injury. Basso, Beattie and Bresnahan (BBB) scores for recovery of locomotor performance revealed a significant improvement in functional recovery at 14 and 28 days post injury in VCE-treated animals compared with the sham-operated group ($n=6$ /group, * $P<0.05$; nonparametric Kruskal-Wallis test).

are particularly susceptible to peroxidation by reactive oxygen species. The primary reactive oxygen species generated in the body is superoxide, which is transformed to hydrogen peroxide by SOD. Hydrogen peroxide is then converted into water and molecular oxygen by catalase or GSH-Px. In a previous study, Kayali *et al.*,² found evidence of oxidative stress and increased GSH-Px activity but unchanged SOD activity in rat spinal cord tissue obtained on the sixth day after injury. In another similar study of central nervous system injury, fluid percussion brain injury increased protein oxidation as evidenced by reduced levels of SOD.¹⁷ Similarly, in our study, SOD had decreased but GSH-Px had increased in SCI rats 7 days after spinal cord contusion. The reason for the disparity between these two enzymes in the contused spinal cord tissue is unclear. However, besides catalyzing the reduction of hydrogen peroxide, GSH-Px may be upregulated in the injured spinal cord by increased production of lipoperoxides from reactive oxygen species-mediated oxidation of polyunsaturated fatty acids in the damaged cord tissue. Hence, lipid peroxidation resulting from upregulation of GSH-Px may have a role in the progression of spinal cord damage following a primary injury.¹⁸ In our study, VCE treatment counteracted all of the observed effects of oxidative stress (SOD and GSH-Px) with an SCI. This phenomenon indicates that VCE treatment influenced the activities of antioxidant enzymes in the acute stage after an SCI. Nevertheless, the roles played by SOD and GSH-Px in the neuroprotection by VCE in SCIs need to be explored further.

VCE treatment inhibits apoptosis and enhances autophagy

Traumatic injury from a cord contusion gives rise to apoptosis and necrosis of neurons and glia. Recently, we demonstrated that after a spinal cord contusion injury in rats, levels of LC3-II, a marker of autophagy, were elevated in neurons near the lesion epicenter.⁶ Liu *et al.*,¹³ provided the time course of apoptosis after a contusion injury. By 4 h, apoptosis was detected in neurons within the lesion area, and this neuronal apoptosis peaked at 8 h. A second wave of apoptotic cells, identified as oligodendrocytes, was detected throughout the white matter at 7 days, associated with degenerating axons. In our previous study, we reported that the expression of

autophagy peaked at 2 h and subsequently had declined to near-normal levels by 1 week. Hence, combined treatment with VCE over 7 days after injury was used in the current study.

There are three main types of cell death: apoptotic, necrotic, and autophagic cell death. The necrotic and apoptotic cell death pathways are known to occur after a human SCI. In our previous study, we proved the occurrence of autophagy after an SCI. Autophagy is a regulated process that is activated for the bulk removal of cellular proteins and organelles. Proteins are transported to membrane-enclosed vesicles for autophagic degradation. Vesicles are fused with lysosomes, which form autolysosomes, and their contents are degraded by lysosomal enzymes. Recent evidence supports a protective role of the lysosomal compartment, as part of the oxidative stress response.¹⁹ Protective effects of VCE against apoptotic cell death are well described in the literature: VCE diminishes homocysteine thiolactone-induced apoptosis in human promyeloid HL-60 cells.⁸ Combined treatment with VCE reduced caspase-9 and -3 activities and myocyte apoptosis.⁹ Conversely, several studies documented enhanced effects of VCE on autophagy. In isolated rat hepatocytes, vitamin E increased autophagy by accelerating LC3 conversion.¹⁰ In human astrocyte glial cell culture, vitamin C accelerated the degradation of intra- and extracellular proteins targeted to the lysosomal lumen by autophagic pathways.¹¹ In our previous study, promotion of autophagy by rapamycin treatment contributed to the functional recovery of rat SCIs.⁷ In this study, after 7 days of combined treatment with VCE, apoptosis after the SCI was suppressed, and autophagy after the SCI was enhanced. These results were congruous with improved functional recovery.

VCE significantly improve locomotor recovery

These two essential micronutrients, VCE, have major antioxidant functions and show beneficial effects in many insults to cells and tissues. Deficiencies of these micronutrients were characterized and shown to cause severe central nervous system (brainstem and spinal cord) damage.²⁰ New evidence revealed that supplementation with VCE may protect against dementia and improve cognitive function in later life.²¹ VCE supplementation also reduces oxidative stress and improves antioxidant enzymes and positive muscle work in chronically loaded muscles of aged rats.²² In the literature review, combined use of VCE in different SCI studies result in different functional outcomes. Cristante *et al.*,¹⁴ did not find any functional improvements in their animals after the combined use of VCE. In comparison to our study, Cristante *et al.*¹⁴ set their contusion injury model at 25 mm impactor releasing height, which cause more severe impaction than our 12.5 mm height. In addition, the drug doses were only half of ours even though their treatment period was longer. These differences may contribute to no functional improvement in Cristante's study. In another study using compression injury model and different drug doses, Robert *et al.*²³ demonstrated the efficacy of antioxidants in functional recovery of spinal cord injured rats and concluded that administration of alpha-tocopherol (that is, vitamin E) enhances the reparative effects against SCI and it seems to be more effective than ascorbic acid (that is, vitamin C). Following administration of the combination of VCE in rat contusion SCIs, the inflammatory response was less intense.¹⁴ Antioxidant therapy after a traumatic SCI in adult rats also showed increased amounts of preserved tissues.²⁴ In our study, the functional recovery after an SCI in rats showed significant improvements after treatment with VCE, as evidenced by modulation of oxidative stress, inhibition of apoptosis, and enhancement of autophagy.

CONCLUSIONS

The present investigation demonstrated the value of assessing drugs that are already available for clinical application in experimental models of SCI. This 'off-the-shelf' strategy has focused on determining potential therapeutic benefits of two micronutrients, VCE, in a clinically relevant model of moderate spinal cord contusion injury. The combined acute administration of both drugs was clearly capable of modulating antioxidative effects, reducing apoptosis, and increasing autophagy at the lesion epicenter, which led to improved functional outcomes.

DATA ARCHIVING

There were no data to deposit.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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