Valeriana officinalis Extracts Ameliorate Neuronal Damage by Suppressing Lipid Peroxidation in the Gerbil Hippocampus Following Transient Cerebral Ischemia

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ABSTRACT As a medicinal plant, the roots of *Valeriana officinalis* have been used as a sedative and tranquilizer. In the present study, we evaluated the neuroprotective effects of valerian root extracts (VE) on the hippocampal CA1 region of gerbils after 5 min of transient cerebral ischemia. Gerbils were administered VE orally once a day for 3 weeks, subjected to ischemia/reperfusion injury, and continued on VE for 3 weeks. The administration of 100 mg/kg VE (VE100 group) significantly reduced the ischemia-induced spontaneous motor hyperactivity 1 day after ischemia/reperfusion. Four days after ischemia/reperfusion, animals treated with VE showed abundant cresyl violet-positive neurons in the hippocampal CA1 region when compared to the vehicle or 25 mg/kg VE-treated groups. In addition, the VE treatment markedly decreased microglial activation in the hippocampal CA1 region 4 days after ischemia. Compared to the other groups, the VE100 group showed the lowest level of lipid peroxidation during the first 24 h after ischemia/reperfusion. In summary, the findings in this study suggest that pretreatment with VE has protective effects against ischemic injury in the hippocampal pyramidal neurons by decreasing microglial activation and lipid peroxidation.

KEY WORDS: • gerbil ischemia • hippocampus • lipid peroxidation • *Valeriana officinalis*

INTRODUCTION

CEREBRAL ISCHEMIA IS a global problem, not only as a cause of disability and death but also due to the enormous financial cost associated with its treatment.¹ Cerebral ischemia, induced by the blockage of blood flow to the brain, results in neuronal death in certain vulnerable brain regions such as the neocortex, striatum, and hippocampus.^{1–3} One of the most important functions of the hippocampus is its role in learning and memory formation.⁴ Transient ischemia-induced neuronal death in the hippocampus results in cognitive impairment.⁵

Mongolian gerbils are commonly used as a model for transient forebrain ischemia due to their incomplete posterior communicating arteries.⁶ The transient interruption of blood flow deprives the brain of oxygen and blood, and the subsequent ischemia–reperfusion injury can result in cell

death.³ The neuronal damage caused by ischemic injury is mediated by excitotoxicity, oxidative stress, inflammation, and apoptosis.^{7–9}

Valeriana is a major genus in the family Valerianaceae, and valerian root extracts (VE) have been used as a traditional herbal medicine for centuries.¹⁰ In particular, VE has been used as a sedative and for the treatment of anxiety and sleep disorders.^{11,12} Many functional components of VE have been reported, including the well-known γ -aminobutyric acid (GABA).¹³ In a recent study, VE exhibited antioxidant effects and decreased lipid peroxidation induced by quinolinic acid.¹⁴ In addition, VE has been reported to have neuroprotective effects in several neurodegenerative diseases such as Parkinson's and Alzheimer's disease.^{15–17} VE was shown to influence the movement of calcium ions and lipid peroxidation after neuronal injury induced by A β in a model of Alzheimer's disease.¹⁶

There are very few reports on the effects of VE against ischemic damage. In this study, we investigated the neuroprotective effects of VE and the possible mechanisms by which it exerts those effects in the hippocampal CA1 region using a gerbil model of transient cerebral ischemia.

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MATERIALS AND METHODS

Experimental animals

Eighty male gerbils were purchased from Japan SLC, Inc. (Shizuoka, Japan). They were housed in animal facilities maintained at 23°C with 60% humidity and a 12-h light–12-h dark cycle with free access to food and tap water. Animal handling and care conformed to guidelines established by current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985, revised 1996) and were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (SNU-100712-1). All experiments and procedures were designed to minimize the number of animals used and the suffering caused by the study procedures.

VE treatment

VE (Naturex Co. Ltd., Avignon, France) was extracted from a 70% ethanol solution. The extract contained valerenic acid at a concentration of 3.401 ± 0.066 mg/g.¹⁸ It has been reported that treatment with 450 and 900 mg VE causes hypnotic effects in people,¹⁹ but the dosage used in the present study was consistent with a previous study in mice.¹⁸

The animals were divided into four groups as follows: (1) sham-operated (Control group), (2) vehicle-treated ischemia (Vehicle group), (3) 25 mg/kg VE-treated ischemia (VE25 group), and (4) 100 mg/kg VE-treated ischemia group (VE100 group). Vehicle or VE was orally administered to gerbils once a day for 3 weeks before the ischemic surgery.

Induction of transient forebrain ischemia

The animals were anesthetized with a mixture of 2.5% isoflurane (Baxter, Deerfield, IL) in 33% oxygen and 67% nitrous oxide. Bilateral common carotid arteries were isolated and occluded using nontraumatic aneurysm clips. The complete interruption of blood flow was confirmed by observing the central artery in retinae using an ophthalmoscope. After 5 min of occlusion, the aneurysm clips were removed from the common carotid arteries. The body temperature under free-regulating or normothermic ($37^\circ\text{C} \pm 0.5^\circ\text{C}$) conditions was monitored with a rectal temperature probe (TR-100; Fine Science Tools, Foster City, CA, USA) and was maintained using a thermometric blanket before, during, and after the surgery until the animals completely recovered from anesthesia. Thereafter, animals were kept on a thermal incubator (Mirae Medical Industry, Seoul, South Korea) to maintain their body temperature until euthanization.

Spontaneous motor activity

To check the effect of VE on hyperactivity after ischemic damage, the spontaneous motor activity was measured in the gerbils before and 1 day after ischemia/reperfusion. Gerbils were individually placed in a Plexiglas cage ($25 \times 20 \times 12$ cm), located inside a soundproof chamber. The locomotor activity was recorded with the Photobeam Activity System-Home Cage (San Diego Instruments, San Diego, CA, USA). Spontaneous motor activity was monitored for 60 min. The number

of times each animal reared and the time (in seconds) spent in grooming behavior were also recorded. Scores were generated from live observations, and video sequences were used for subsequent reanalysis.

Tissue processing

Four days after ischemia/reperfusion, the animals in the Control, Vehicle, VE25, and VE100 groups ($n=5$ in each group) were anesthetized with 30 mg/kg Zoletil 50® (Virbac, Carros, France) and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4), followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brains were removed and fixed in the same fixative for 8 h and then dehydrated with graded concentrations of alcohol before being embedded in paraffin. The paraffin-embedded tissues were sectioned on a microtome (Leica Microsystems GmbH, Wetzlar, Germany) into 3- μm coronal sections and mounted onto silane-coated slides.

Cresyl violet staining and neuronal quantification

The brain tissue sections were stained with cresyl violet acetate (CV; Sigma, St. Louis, MO, USA). CV was dissolved at 1.0% (w/v) in distilled water with glacial acetic acid (Sigma). Before and after staining for 2 min at room temperature, the sections were washed twice in distilled water. They were then dehydrated and mounted using Canada Balsam (Kanto, Tokyo, Japan). CV-positive neurons were identified in all groups using an image analysis system equipped with a computer-based charge-coupled device (CCD) camera (software: Optimas 6.5; CyberMetrics, Scottsdale, Arizona). The number of CV-positive neurons was counted in a 250×250 μm square applied, approximately, at the medial, central, and lateral parts of the CA1 region in the stratum pyramidale (SP). Images of all CV-positive structures in the hippocampal CA1 region were captured using a BX51 light microscope (Olympus, Tokyo, Japan) equipped with a digital camera (DP71; Olympus). Cell counts were obtained by averaging the counts from the sections taken from each animal and the numbers were presented as a percentage of control.

Immunohistochemistry

To ensure that the immunohistochemical data were comparable between groups, the sections were carefully processed under the same conditions. The tissue sections were selected between 1.4 and 2.0 mm posterior to the bregma, with reference to a gerbil atlas.²⁰ The sections were hydrated and treated with 0.3% hydrogen peroxide (H_2O_2) in PBS for 30 min. For antigen retrieval, the sections were placed in 400-mL jars filled with a citrate buffer (pH 6.0) and heated in a microwave oven (Optiquick Compact; Moulinex, Bagnolet Cedex, France) operating at a frequency of 2.45 GHz and an 800-W power setting. After three heating cycles of 5 min each, slides were allowed to cool at room temperature and were washed in PBS. After washing, the sections were first incubated in 10% normal goat serum in PBS for 30 min. They were then incubated with a diluted rabbit anti-Iba-1 antibody

(1:500; Wako, Richmond, VA, USA) for 48 h at 4°C. Finally, they were incubated with biotinylated goat anti-rabbit IgG and streptavidin–peroxidase complex (diluted 1:200, Vector Laboratories, Inc., Burlingame, CA, USA) and then visualized with 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA) in 0.1 M Tris-HCl buffer (pH 7.4).

Measurement of lipid peroxidation

To determine the protective mechanism of VE against ischemic damage, 4-hydroxy-2-nonenal (HNE) was used as an indicator of lipid peroxidation. Animals in the Vehicle, VE25, and VE100 groups ($n=20$ in each group) were anesthetized with 30 mg/kg Zoletil 50® (Virbac, Carros, France) at 0, 3, 12, and 24 h after ischemia/reperfusion ($n=5$ at each time point). The hippocampi were removed and used for measurement of HNE (Bioxytech HAE-586 spectrophotometric assay kit; OxisResearch, Portland, OR, USA) levels. The hippocampi were homogenized in a buffer containing 10 mM HEPES (pH 7.5), 200 mM mannitol, 70 mM sucrose, 1 mM EGTA, and 5 mM butylated hydroxytoluene. One milliliter of homogenate was extracted with dichloromethane, and an aliquot of the lower organic phase was dried under nitrogen and reconstituted with water. The sample was mixed with *N*-methyl-2-phenylindole in acetonitrile and methanesulfonic acid, the mixture was incubated and centrifuged, and the absorbance of the clear supernatant was determined at 586 nm using a Beckman DU-64 spectrophotometer (Beckman, Fullerton, CA, USA). The HNE concentrations in the experimental samples were determined against HNE standards provided in the assay kit.

Statistical analysis

The data presented represent the means of the experiments performed for each experimental investigation. The differences among the means were statistically analyzed by a one-way or two-way analysis of variance followed by a Bonferroni's post-hoc method in order to compare the effects of VE against ischemic damage.

RESULTS

Effects of VE on ischemia-induced hyperactivity

We evaluated the effect of VE by assessing the locomotor activity in the sham-operated (Control) and VE-treated groups, both before and 1 day after ischemia/reperfusion. In the Vehicle and VE25 groups, the locomotor activity was significantly increased (2.54- and 2.40-fold, respectively) 1 day after ischemia compared to the Control group. In the VE100 group, the locomotor activity was significantly lower by 1.46-fold compared to the Vehicle group (Fig. 1).

Effects of VE on neuronal damage

In the Control group, CV-positive pyramidal cells were easily detected in the CA1 region 4 days after the sham operation (Fig. 2A). In the Vehicle group, few CV-positive cells were observed in the CA1 region 4 days after ischemia/reperfusion due to the delayed neuronal death following

FIG. 1. Locomotor activity in the sham-operated (Control), vehicle-treated (Vehicle), 25 mg/kg VE-treated (VE25), and 100 mg/kg VE-treated (VE100) groups. The spontaneous locomotor activity was observed 1 day after ischemia/reperfusion and evaluated in terms of entire distance (meters) traveled in 60 min ($n=5$ per group, $*P<.05$ vs. the Control group; $*P<.05$ vs. the Vehicle group; $†P<.05$ vs. the VE25 group). The bars indicate the mean $\pm$ SE. VE, valerian root extracts.

transient cerebral ischemia (Fig. 2B). In this group, the number of CV-positive cells was 1.8% of the Control group (Fig. 2E). In the VE25 group, the number of CV-positive cells was slightly higher compared to the Vehicle group (Fig. 2C, E). In the VE100 group, CV-positive cells were abundantly detected in the CA1 region and were significantly higher when compared to the Vehicle and VE 25 groups (Fig. 2D). In this group, the number of CV-positive cells was 53.9% of the Control group (Fig. 2E).

Effects of VE on reactive microgliosis

In the Control group, Iba-1-positive cells had small cytoplasm with thin processes (Fig. 3A). In the Vehicle group, Iba-1-positive cells had hypertrophied cytoplasm with bushy processes (reactive form) (Fig. 3B). In addition, some of the Iba-1-positive cells had round cytoplasm with very short processes (phagocytic form). In the VE25 group, many Iba-1-positive cells had hypertrophied cytoplasm with long processes (Fig. 3C). In the VE100 group, many Iba-1-positive cells showed a similar morphology to cells in the Control group (Fig. 3D).

Effects of VE on lipid peroxidation

At 0 h after ischemia/reperfusion, there was no significant difference in HNE levels among the three groups. At 3 h postischemia/reperfusion, HNE protein levels were at a peak in all groups, and the levels of HNE in the VE100 group were significantly lower than those in the Vehicle and VE25 groups. HNE levels decreased with time after ischemia/reperfusion. In addition, HNE levels in the VE25 group were lower than those in the Vehicle group at 12 and 24 h after ischemia/reperfusion, and the VE100 group showed the lowest levels of HNE among all the groups (Fig. 4).

FIG. 2. Cresyl violet staining in the CA1 region in the Control (A), vehicle-treated (Vehicle; B), 25 mg/kg VE-treated (VE25; C), and 100 mg/kg VE-treated (VE100; D) groups at 4 days after ischemia/reperfusion. Scale bar = 50 μ m. (E) Relative analysis of the cresyl violet-positive cell number ($n = 5$ per group, $*P < .05$ vs. the Control group; $^{##}P < .05$ vs. the Vehicle group; $^{\dagger}P < .05$ vs. the VE25 group). The bars indicate the mean $\pm$ SEM. Color images available online at www.liebertpub.com/jmf

FIG. 3. Immunohistochemistry for Iba-1 in the CA1 region of the Control (A), vehicle-treated (Vehicle; B), 25 mg/kg VE-treated (VE25; C), and 100 mg/kg VE-treated (VE100; D) groups. Scale bar = 50 μ m. Color images available online at www.liebertpub.com/jmf

FIG. 4. HNE levels in the hippocampi of vehicle-treated (Vehicle), 25 mg/kg VE-treated (VE25), and 100 mg/kg VE-treated (VE100) groups at various time points after ischemia/reperfusion. HNE levels in the VE100 group are lower than those in the corresponding Vehicle or VE25 groups ($n=5$ per group, $*P<.05$ vs. the Vehicle group; $#P<.05$ vs. the VE25 group). The bars indicate the mean $\pm$ SEM. HNE, 4-hydroxy-2-nonenal.

DISCUSSION

In transient cerebral ischemia, ischemic damage induces delayed neuronal death in the CA1 pyramidal cell layer of the hippocampus.^{2,21} During ischemic injury, various factors such as energy imbalance, neuroinflammation, and oxidative stress trigger delayed neuronal death.^{22–24}

Transient cerebral ischemia induces not only neuronal death but also behavioral impairment.^{25,26} Transient hyperactivity is frequently observed in gerbils after ischemia.^{27,28} The disruption of neurotransmission following ischemia induces motor hyperactivity, and it has been reported that neuroprotective agents could ameliorate ischemia-induced hyperactivity.^{29,30} In the present study, we observed that VE treatment protected hippocampal neurons from ischemic damage and restored behavioral impairment in a spontaneous motor activity test.

Energy failure during cerebral ischemia disrupts cellular homeostasis and generates excessive reactive oxygen species (ROS).^{31,32} The ROS-mediated redox state activates genes related to inflammation and cellular defense.³³ It has been reported that the ethyl acetate extract of *V. officinalis* and its main chemical components, including acetylvalerenolic acid and valerenic acid, inhibit the nuclear factor (NF)- κ B activity in cell culture systems.³⁴ Microglia are activated due to the immune response following cerebral ischemia.³⁵ In a previous study, we observed that Iba-1, which is specifically expressed in microglia, was significantly increased after ischemia/reperfusion.²³ In ischemic brain injury, activated microglia produce various proinflammatory cytokines and recruit inflammatory cells.³⁶ Consequently, excessive activation of microglia induces detrimental and neurodegenerative effects on neurons after ischemia/reperfusion.^{37,38} In addition, we observed that treatment with VE decreased neuronal loss in the CA1 region of the gerbil hippocampus. It has been reported that the effects of valerian and its active

component, valerenic acid, are closely related to the GABA system.^{13,39} GABA-induced depolarization activates the cAMP response element-binding signaling, which promotes neuronal survival by activating downstream survival genes.⁴⁰ Apart from the connection between valerian and the GABA system with regard to neuroprotection, we also found that VE is effective at preventing Iba-1-positive microglia from being activated. This may be related to the inhibitory effect of the VE components acetylvalerenolic acid and valerenic acid on NF- κ B.³⁴ Based on the results of the present study, we suggest that the inhibitory effect of VE on microglia might protect hippocampal CA1 neurons from ischemic damage.

Increased ROS after ischemia/reperfusion accelerates lipid peroxidation and results in cellular damage. The production of lipid peroxides is increased in vulnerable brain regions, especially in the striatum, cortex, and hippocampus.^{41,42} In our previous study, we demonstrated that VE decreased lipid peroxidation in a model of aging induced by D-galactose.¹⁸ In the present study, HNE levels peaked at 3 h after ischemia/reperfusion and decreased with time. We observed that VE treatment lowered HNE levels, and we hypothesize that this might contribute to the increased survival of pyramidal neurons in the gerbil hippocampus. This result supports the previous observation that VE ameliorates the reduction of total thiol content in homogenates as well as decreased H₂O₂ production in mitochondria of flies exposed to rotenone.⁴³

In conclusion, administration of VE significantly increases neuronal survival after ischemia/reperfusion by decreasing reactive microglia and the levels of lipid peroxidation.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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