Protective Effects of Ginseng Components in a Rodent Model of Neurodegeneration

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To test the proposed neuroprotective activity of whole ginseng extract and its components, we used 3-nitropropionic acid (3-NP), an inhibitor of succinate dehydrogenase, to produce neurodegeneration. Treatment with 3-nitropropionic acid (90mg/kg) over a 5-day period resulted in severe impairment of movement and loss of neurons in the striatum. Pretreatment with a preparation from the whole root of American ginseng had no protective effects. Pretreatment with a preparation of ground leaves and stems, which contains greater levels of ginsenosides than ground root, improved the behavioral score and reduced the volume of the striatal lesion. A partial purification of American ginseng was performed to concentrate the putative protective components: Rb_1, Rb_3, and Rd (termed Rb extract). Pretreatment with the Rb extract significantly reduced the 3-nitropropionic acid–induced motor impairment and cell loss in the striatum, and it completely prevented any mortality. Significant effects on motor function, mortality, and the striatal lesion volume also were measured in animals pretreated with the individual ginsenosides, Rb_1, Rb_3, or Rd. The results demonstrate that some of the ginsenosides have neuroprotective activity, and that a partial purification of whole ginseng to concentrate the neuroprotective components may have utility as a neuroprotective agent.

Ann Neurol 2005;57:642–648

The three most commonly used species of ginseng are *Panax ginseng* (Asian), *Panax quinquefolius* (American), and *Panax japonicus* (Japanese).[1] Whereas some other constituents of the plant extract may have some activity, the ginsenosides are considered to have the most activity.[2] The ginsenosides are thought to have antineoplastic, antistress, and antioxidant properties. Most experimental work with this herbal product has been done with the whole extract, and the effects of the individual ginsenosides are just beginning to be determined.

More than 28 ginsenosides have been isolated from American ginseng, and they have been divided roughly into two groups based on structure. The panaxadiols include Rb_1, Rb_2, Rb_3, Rc, Rd, Rg_3, Rh_2 and Rh_3, whereas the panaxatriols include Re, Rf, Rg_1, Rg_2, and Rh_1. Rb_1 often is used to represent the panaxadiols, whereas Rg_1 represents the panaxatriols. The different species of ginseng have been shown to have different relative amounts of panaxadiols and panaxatriols. For example, American ginseng generally contains a smaller Rg_1/Rb_1 ratio compared with Asian ginseng. Given the evidence that Rb_1 has neuroprotective activity both in vivo and in vitro,[3–6] the smaller ratio of Rg_1/Rb_1 might explain the "cool" characteristic of American ginseng. The larger ratio of Rg_1/Rb_1 in Asian ginseng may explain the so-called warm property.[7] For this study of the neuroprotective activity of ginseng and its components, a smaller Rg_1/Rb_1 ratio would be predicted to be more effective. Therefore, American ginseng was used in this study.

To test the neuroprotective activity of ginseng and it components, we chose 3-nitropropionic acid (3-NP), a succinic dehydrogenase inhibitor. Administration of 3-NP models conditions in which there is an interruption in energy metabolism, followed by cell death.[8,9] Specifically, 3-NP has been used to model the neurodegeneration that occurs in Huntington's disease.[10,11] In this study, the protective effects of whole-ginseng preparations from root and from leaves and stems and a partial purification of the leaves and stems (to enrich the Rb ginsenosides, called the Rb extract) were tested against 3-NP neurotoxicity using both behavioral changes and markers of neuronal damage. Because the Rb extract had significantly more neuroprotective activity than the whole-ginseng preparations, the individual ginsenosides in the Rb extract also were tested to determine their contribution to the neuroprotective activity.

Materials and Methods

Ginseng extracts from the root and from the leaves and stems of American ginseng, with analysis, were purchased

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Received Dec 13, 2004, and in revised form Feb 13, 2005. Accepted for publication Feb 24, 2005.

Published online Apr 25, 2005, in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/ana.20450

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Table 1. Characteristics of Ginseng Products

Characteristic	Total Ginsenosides	Rb_1	Rb_3	Rd	Total Rb_1, Rb_3, Rd	Percentage of Each gm That Is in Rb Group
Root	30.2%	2.4%	14.6%	9.9%	26.9%	8.1%
Leaves/stems	85.2%	1.9%	19.2%	14.7%	35.8%	30.5%
Rb extract	94.7%	24.8%	46.4%	13.1%	84.3%	80.0%
Rb_1	100%	94.6%				
Rb_3	100%		90.3%			
Rd	100%			90.9%		

The final column shows the calculation of the % of each gram of powder that is Rb_1, Rb_3, or Rd (ie, the major components of the Rb extract).

from Jilin Ginseng (China; more information is available via the internet at www.ginseng99.com). The single ginsenosides (Rb_1, Rb_3 and Rd) were obtained from Hongjiu Biotech (Jilin City, China). The levels of Rb_1, Rb_3, and Rd in the ginseng extracts and the single ginsenosides were determined by thin-layer chromatography and high-performance liquid chromatography (column: Adsorbosphere XL-C-18B 90A (Alltech Associates, IL), 5μm; inner diameter, 250 × 4.6mm; flow phase: CH_3CN, 0.06% trifluoroacetic acid in H_2O; flow rate, 0.3ml/min; detection: ultraviolet at 203 nm; Table 1). The partial purification of the leaves and stems to produce the Rb extract was performed as described previously.[12]

All animal experiments were performed in accordance with the National Institutes of Health (NIH) guide for the care and use of laboratory animals (NIH publication no. 8023, revised 1996) and with the approval of the local animal use committee. Adult male Sprague–Dawley rats weighing 290 to 330gm received either 3-NP treatment alone or pretreatment with one of the ginseng preparations 1 hour before each dose of 3-NP (n = 7–8 for each). Another three animals (control animals) received matching volumes of normal saline. There was no difference in the animals that received normal saline followed by 3-NP and those that received 3-NP alone; therefore, the results from these animals were combined. 3-NP was dissolved in saline, and the pH was adjusted to 7.4 with NaOH. Preliminary experiments using the dosing schedule reported in the literature[13] (30mg/kg/day intraperitoneally for 3 days) resulted in a nearly two-thirds mortality rate in the experimental group. Therefore, for these experiments, animals received 30mg/kg on day 1, and then 15mg/kg/day for the next 4 days. This schedule resulted in the same total dose of 3-NP (90mg/kg), but with a lower mortality rate. Both 3-NP and the ginseng preparations were given intraperitoneally.

The Rb extract contains the ginsenosides of interest (Rb_1, Rb_3 and Rd) at the 80% level. To determine the doses of root and of leaves and stems preparations to test, we considered the relative concentrations of total ginsenosides and ginsenosides in the Rb group. For the ginseng root preparation, doses of 66 and 132mg/kg include total ginsenosides at 20 and 40mg/kg and Rb ginsenosides at 5.4 and 10.8mg/kg, respectively. For the leaves and stems preparation, doses of 25 and 50mg/kg include total ginsenosides at 20 and 40mg/kg and Rb ginsenosides at 7.2 and 14.3mg/kg, respectively. For the individual ginsenosides, the doses equivalent to the dose of each in the Rb extract at 10mg/kg were used: Rb_1 at 2.5mg/kg, Rb_3 at 5.0mg/kg, or Rd at 1.25mg/kg.

Behavior in all groups was recorded daily and immediately before death. Behavior was graded 0 through 5 according to the scale described previously[14]: grade 0, normal behavior; grade 1, general slowness in movement because of mild hind-limb impairment; grade 2, prominent gait abnormality with poor coordination; grade 3, nearly complete hind-limb paralysis; grade 4, inability to move because of four-limb impairment; and grade 5, recumbency or death.

Severe neuronal stress in the brain was assessed using immunoreactivity for heat shock protein 72 (HSP72).[15] Neuronal damage was estimated using Nissl stain. Animals were deeply anesthetized and perfused through the heart with 4% buffered paraformaldehyde 48 hours after the last injection of 3-NP. Brains were fixed overnight. Coronal sections (50μm) were cut with a Vibratome (Technical Products, St. Louis, MO). After blocking, the sections were processed for immunolabeling with anti-HSP72 (1:2,000; mouse monoclonal antibody; Oncogene Research Products, Boston, MA) overnight at 4°C, followed by biotinylated goat anti–mouse antibody (1:200; Vector Laboratories, Burlingame, CA), and visualized with 3-3′-diaminobenzidine (Vector Laboratories). Adjacent sections were stained with cresyl violet.

In all animals, the area of HSP72-positive staining corresponded to the area of neuronal loss on the sections with cresyl violet staining, indicating that the remaining neurons in the region were severely stressed. Because the HSP72 sections had clearer boundaries, the volume of the striatal lesion was estimated using sections stained for HSP72 immunoreactivity using NIH Image J software (Version 1.30; NIH, Bethesda, MD). Every 10th section through the entire striatum was stained for HSP72 to determine the extent of the lesion. At least six sections evenly spaced through the lesion volume were measured for each animal. The actual distance between sections used for measurements depended on the extent of the lesion. The lesion volume was estimated using the following formula: volume = $(a_1 + a_2, + \ldots + a_n)/n \times d$, where d = distance (in millimeters) between sections, and a_1, a_2, a_3, … = area (in square millimeters) of the lesion for individual sections.[16]

All data are expressed as means ± standard error of the mean, and differences were analyzed using a one-way analysis of variance followed by Bonferroni post hoc test. $p < 0.05$ was considered statistically significant.

Table 2. Effects of Ginseng on 3-NP–Induced Motor Dysfunction

Treatments	N[a]	Grade[b]	Grade ≥1	Mortality
3-NP alone	8	3.8 ± 0.3	8/8	2/8
TG-Rt				
66mg/kg	8	4.0 ± 0.7	8/8	4/8
132mg/kg	7	3.8 ± 0.5	7/7	3/7
TG-LS				
25mg/kg	7	4.4 ± 0.4	7/7	4/7
50mg/kg	8	2.5 ± 0.6[c]	6/8	2/8
Rb extract				
5mg/kg	8	1.1 ± 0.4[d,e]	5/8	0
10mg/kg	8	0.8 ± 0.3[d,e]	4/8	0
20mg/kg	8	0.8 ± 0.3[d,e]	4/8	0
40mg/kg	7	1.0 ± 0.4[d,e]	3/7	0
Rb_1				
2.5mg/kg	7	0.71 ± 0.35[d,e]	3/7	0
Rb_3				
5mg/kg	8	1.56 ± 0.31[d]	6/8	0
Rd^-				
1.25mg/kg	7	1.64 ± 0.64[d]	5/7	1/7

[a]Number of animals in each group; [b]Grade of motor function 24 hours after the last dose of 3-NP (see Materials and Methods for details); [c]$p <$ 0.01 compared with 3-NP or TG-Rt; [d]$p < 0.001$ compared with 3-NP alone; [e]$p < 0.001$ compared with all doses of TG-LS or TG-Rt.
NP = nitropropionic acid; TG-Rt = total ginsenosides from the root; TG-LS = total ginsenosides from the leaves and stems.

Results

A reduction in motor function was first noted in the observation period before the third dose of 3-NP alone (n = 8). At 48 hours after the last dose, there was complete hind-limb paralysis (grade 4) or recumbency (grade 5) in approximately half of the surviving animals. In addition, one of the eight animals died after the fourth dose, and another one died after fifth dose; thus, the overall mortality rate is 25% (Table 2). All surviving animals treated with 3-NPA alone had massive cell loss in the striatum as shown with Nissl staining (Fig 1). This area with cell loss also had immunoreactivity for HSP72 in the cells that remained (Fig 2). In animals treated with a total of 90mg/kg 3-NP alone, the mean estimated volume of the lesion in the striatum was 23.3 ± 3.8mm^3 (Fig 3). In four animals (50%) treated with 3-NP alone, there also was significant damage in the hippocampus (see Fig 2).

Pretreatment with total ginsenosides from the root (TG-Rt, 66 and 132mg/kg) or total ginsenosides from the leaves and stems (TG-LS, 25mg/kg) had no effect on the 3-NP–induced motor impairment or mortality (see Table 2). All animals that died had a behavioral grade of 4 to 5. The survivors in these groups were scored grades 2 to 4 at 24 hours after the last dose of 3-NP. Pretreatment with TG-LS at 50mg/kg significantly reduced the motor impairment compared with 3-NP alone. There was a trend toward a decrease in mortality that did not reach statistical significance (χ^2 test). The striatal lesion in the animals that received either dose of the root extract (n = 7 survivors; see Fig 3) was 19.4 ± 3.2mm^3, which was not statistically different from the lesion in the 3-NP alone group. In this group, four of the seven animals also had immunoreactivity for HSP72 in the hippocampus. The striatal lesion in the animals who had received either dose of the leaves and stems preparation (n = 9 survivors) was 8.2 ± 2.7mm^3, which was statistically different from the 3-NP alone group ($p < 0.05$). In this group, three of the nine survivors had immunoreactivity for HSP72 in the hippocampus.

Pretreatment with the Rb extract reduced the 3-NP–induced behavioral changes and striatal lesions and completely prevented mortality (see Table 2 and Figs 1–3). After the second dose of 3-NP, all animals pretreated with 5, 10, 20, or 40mg/kg ginsenosides showed normal behavior. With additional doses of 3-NP, some animals pretreated with the Rb extract showed mild impairment of behavior. In the eight animals pretreated with 5mg/kg ginsenosides (intraperitoneally), three behaved normally (grade 0), four were scored grade 1 or 2, and one showed nearly complete hind-limb paralysis (grade 3) after the fifth dose of 3-NP. Only the animals with a behavioral score of 2 or 3 had a measurable striatal lesion. Half of the animals pretreated with 10, 20, or 40mg/kg ginsenosides had no behavioral changes. The remainder were scored grade 1 or 2 after the fifth dose of 3-NP. Only 3 animals in these dose groups (23 total animals) had a measurable striatal lesion. Two of these animals had received 10mg/kg ginsenosides, and one had received 20mg/kg ginsenosides. All three of these animals had a

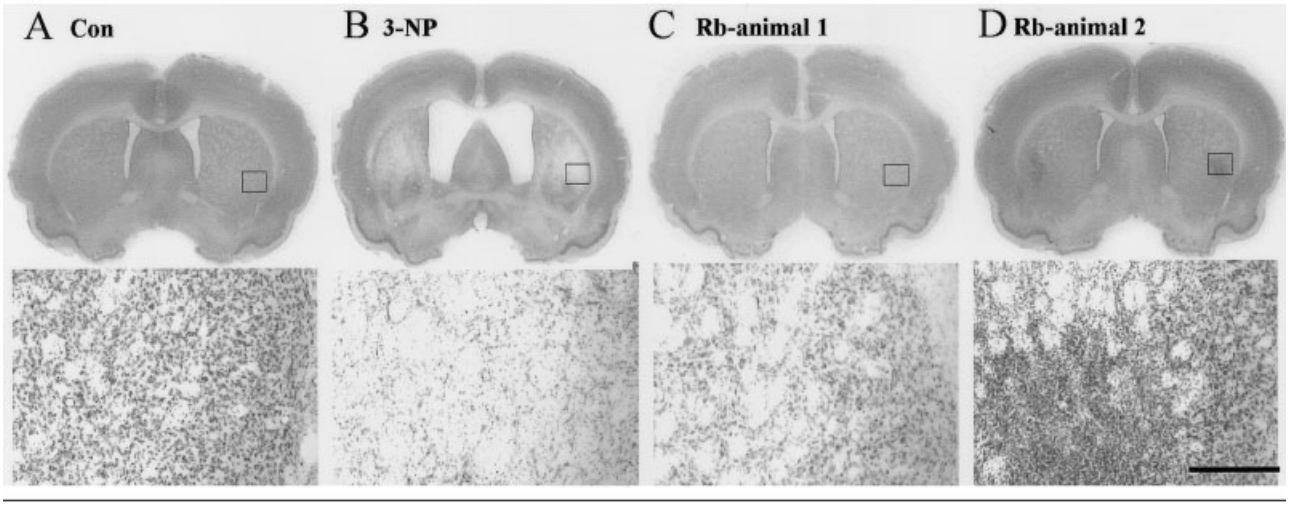

Fig 1. Neuroprotective effect of the Rb extract identified with Nissl staining. Sections from a representative control (Con) animal and an animal treated with 3-nitropropionic acid alone (3-NP) are shown. (C) presents a section from an animal pretreated with 10mg/kg of the Rb extract. This animal had general slowness and is the same animal shown in Figure 2B. (D) presents a section from an animal pretreated with 10mg/kg of the Rb extract that had a behavior score of 2. This is the same animal shown in Figure 2C. For each coronal section, the area indicated by the box is shown at greater magnification just below. Calibration bar = 800μm for the photomicrographs.

motor impairment score of 2. Because so few animals pretreated with the Rb extract had a measurable striatal lesion, the results from all doses were averaged into one group. The estimated volume of the striatal lesion was $0.4 \pm 0.2mm^3$ in animals pretreated with the Rb extract, which is significantly smaller than the lesion after 3-NP alone and after pretreatment with the root preparation. No animal pretreated with any dose of the Rb extract had neuronal loss or evidence of severe neuronal stress in the hippocampus.

Pretreatment with Rb_1 (2.5mg/kg), Rb_3 (5mg/kg), or Rd (1.25mg/kg) significantly reduced the behavioral impairment and striatal lesions induced by 3-NP (see Table 2 and Fig 3). Of the seven animals pretreated with Rb_1, only three had abnormal behavior (grades 1 and 2). In animals pretreated with Rb_3, six of eight had grade 1 or 2 motor impairment. In animals pretreated with Rd, one of seven behaved normally at 24 hours after the last dose of 3-NP, four had grade 1 or 2 motor impairment, and the last animal died. The effects of all three ginsenosides on motor impairment were statistically significant compared with 3-NP alone. Two of seven animals pretreated with Rb_1, seven of eight animals pretreated with Rb_3, and five of six surviving animals pretreated with Rd had immunoreactivity for HSP72 in the striatum. The volume of the striatal lesion was decreased significantly in animals pretreated with Rb_1 ($1.3 \pm 0.8mm^3$), Rb_3 ($10.8 \pm 1.8mm^3$), or Rd ($9.0 \pm 2.7mm^3$). No animal pretreated with an individual ginsenoside (who survived) had evidence of neuronal loss or severe stress in the hippocampus.

Animals treated with 3-NP alone lose weight during the treatment period (Fig 4), and pretreatment with any of the ginseng products did not prevent this weight loss. However, animals treated with 3-NP alone continued to lose weight in the 48 hours after the last dose of 3-NP. This continued weight loss was not altered by pretreatment with either the root (TG-Rt, 132mg/kg) or leaves and stems (TG-LS, 50mg/kg) preparations. However, all animals pretreated with the Rb extract (at all doses) or the individual ginsenosides gained weight during the 48 hours after the last dose of 3-NP.

Discussion

This study demonstrates that some, but not all, components of ginseng have neuroprotective activity. The neuroprotective activity was particularly marked in an extract that was enriched in panaxadiols, specifically Rb_1, Rb_3, and Rd. The data with the individual ginsenosides show that Rb_1, Rb_3, and Rd all contribute to the protective effect, but that Rb_1 makes the largest contribution to the overall effectiveness of the partial extract. This conclusion also is supported by the modest protection with the leaves and stems preparation, which has greater levels of the panaxadiols (35.8% of Rb_1, Rb_3, and Rd) compared with the root preparation (26.9% of Rb_1, Rb_3, and Rd), which had no protective effect. Because the neurodegeneration induced by 3-NP mimics the neuronal loss in Huntington's disease, the results suggest that the Rb extract, or Rb_1 alone, may have clinical utility to prevent neurodegeneration in Huntington's disease or other neurological disorders.

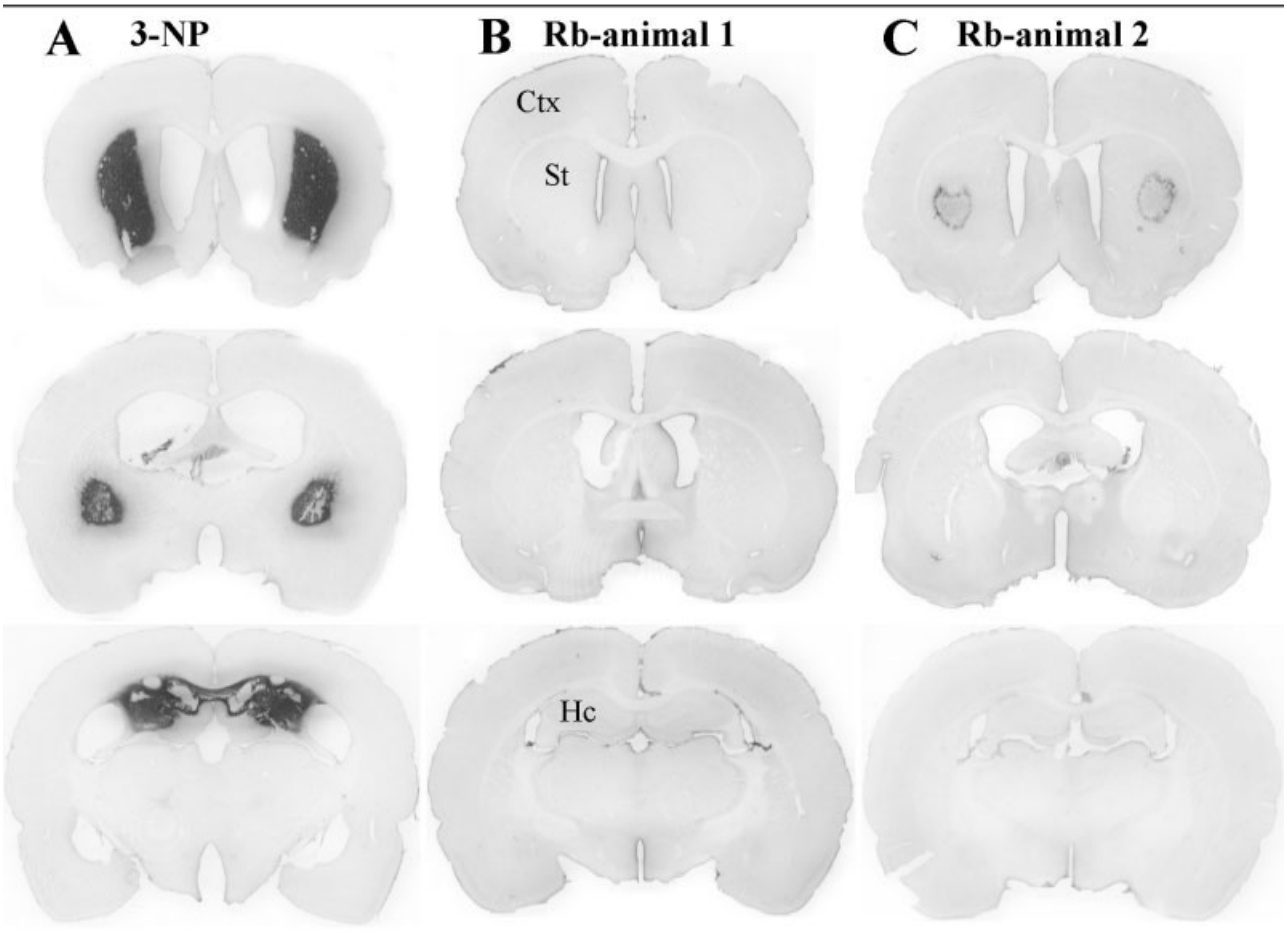

Fig 2. Neuroprotective effect of the Rb extract identified with immunoreactivity for heat shock protein 72 (HSP72). Three coronal sections are presented from three different animals. (A) The animal was treated with 3-nitropropionic acid alone (3-NP) and killed 2 days after the last dose. Positive immunoreactivity for HSP72 is seen easily in the striatum and hippocampus. (B) The animal was pretreated with 10mg/kg of the Rb extract followed by 3-NP, and this animal exhibited general slowness. There was no apparent neuronal damage in this animal. (C) The animal was pretreated with 10mg/kg of the Rb extract. This animal had some behavioral impairment (grade 2), and there was some damage in the striatum, but none in the hippocampus.

The data in this study confirm and extend previous work that also provides evidence of a neuroprotective effect of individual panaxadiol ginsenosides, most notably Rb_1. Pretreatment with the isolated ginsenoside Rb_1 reduces the loss of neurons in the hippocampus in gerbils after bilateral occlusion of the carotid arteries.[3,4] This action is thought to be partially mediated by the scavenging of free radicals by Rb_1.[17] More recent studies have examined the effects of panaxadiols, including Rb_1, in in vitro systems. Rb_1 protects against glutamate-induced excitotoxicity in neuronal cultures.[5,6] Rc has been shown to enhance GABA currents in *Xenopus* oocytes expressing GABA receptors.[18] In additional work, Rb_1 has been shown to have anxiolytic effects,[19] but the mechanism behind this effect is unknown. Rb_1 injected into the lateral ventricle has no effect on basic synaptic transmission.[20] This suggests that Rb_1 is not anxiolytic by suppression of excitatory transmission.

In this model, the Rb extract was maximally effective at 8 to 16mg/kg. Because Rb_1, Rb_3, and Rd are all active components, one would predict that the root or leaves and stems preparations would be equally efficacious at doses that contain the same effective amount of the Rb extract. However, at these doses, the root preparation had no effect on the behavioral changes, mortality, or striatal lesion from 3-NP, and the leaves and stems preparation had only a partial protective effect. These results suggest that other components in these preparations have opposite, or neutralizing, effects compared with the ginsenosides in the Rb extract. The most likely components responsible are the panaxatriol ginsenosides, especially Rg_1, because they are reported to be neuronal stimulants.[7] However, the data

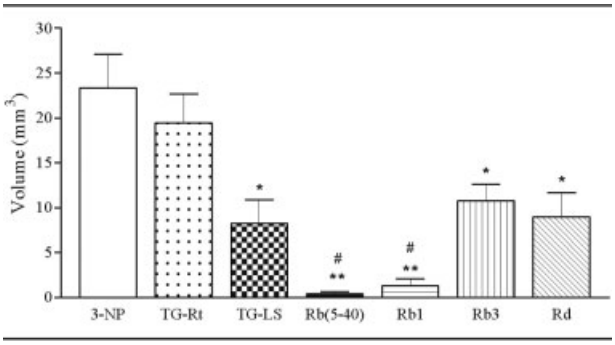

*Fig 3. Effects of ginseng preparations on 3-NP–induced striatal lesions. Striatal lesions were identified by heat shock protein 72 stain, and the volume of each striatal lesion was measured in the survivors in all experimental groups. Data are presented as mean ± standard error of the mean. Because there was little difference across doses, the data from the Rb extract doses were pooled for presentation (n = 31). Because the dose most closely matches the Rb extract and because of a greater survival rate, only the data from the larger dose of root or leaves and stems preparations are presented. The lesion volumes were significantly decreased in all groups that had received the preparations of ginseng, except for treatment with the root preparation (TG-Rt group), compared with 3-nitropropionic acid alone (3-NP; *p < 0.05; **p < 0.001). The mean lesion volume in the group that received Rb extract or Rb_1 was significantly less than that in the TG-Rt groups (#p < 0.01).*

on the panaxatriols are inconsistent with some studies suggesting neuroprotective effects[5,6,21] and other studies showing no effect.[3,4] In addition to the panaxatriols, there could be other unknown components (nonginsenosides) that contribute to these effects. This is especially true for the root preparation, where the nonginsenoside component is the largest.

How does the Rb extract work? Ginsenosides are amphiphilic in nature and have the ability to intercalate into the plasma membrane. This leads to changes in membrane fluidity, which can alter membrane function and secondarily alter membrane receptor function. There is also evidence of interactions with membrane-bound receptors and the possibility of binding to steroid receptors in the cytoplasm leading to changes in gene expression,[2] but this action has not been demonstrated in the central nervous system. The most accepted theory about the mechanism of action is that the ginsenosides (at least some of them) have antioxidant properties and the ability to scavenge free radicals.[17,22] Increased levels of reactive oxygen species and reduced glutathione levels have been measured in vivo and in vitro after exposure to 3-NP.[23,24] These results indicate that the conditions are favorable for oxidative damage early after exposure to 3-NP, which has been suggested to be a prerequisite for striatal lesion formation.[25,26] The postulated ability of the ginsenosides to scavenge free radicals might explain the neuroprotective effects of ginseng and the results of this study, but further research is needed to elucidate the precise mechanism for the neuroprotection. This potential action of Rb ginsenosides suggests that the Rb extract, or Rb_1, may be useful for neuroprotection in other energy disorder–related diseases such as ischemia and Parkinson's disease.[27,28]

This study was supported by the NIH (National Institute of Neurological Disorders and Stroke, NS39941, J.L.S.).

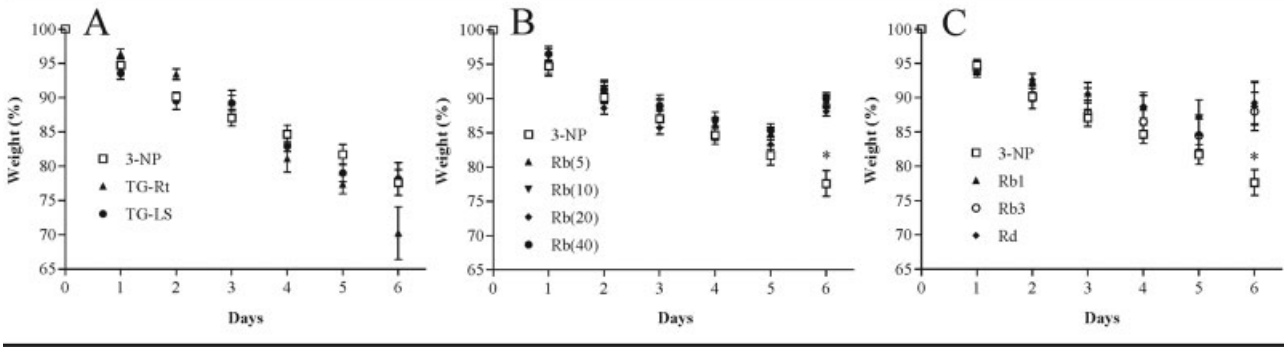

Fig 4. Body weight during and after administration of 3-nitropropionic acid (3-NP). Data are expressed as the percentage body weight relative to the weight obtained immediately before the first dose (day 0) and are shown as mean ± standard error of the mean. (A) The mean body weight for the animals in the 3-NP alone, total ginsenosides from the root (TG-Rt; n = 7), and total ginsenosides from the leaves and stems (TG-LS; n = 9) groups are presented. The results from survivors who received either dose of the root or leaves and stems preparations are pooled. (B, C) The mean body weight for the animals in the 3-NP alone group are repeated, and values for the animals that received the four doses of the Rb extract (B) or were pretreated with the individual ginsenosides (C) are presented. Asterisk *represents a statistically significant difference* (p <0.01) *between all of the treatment groups and the 3-NP alone group (n = 6 for 3-NP alone; n = 7–8 for each dose of each ginseng preparation).*

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