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Ginsenosides attenuate the 3-nitropropioic acid-induced rat striatal degeneration in an age-dependent manner

Jong-Hoon Kim and Seung-Yeol Nah#

Research Laboratory for the Study of Ginseng Signal Transduction and Dept. of Physiology, College of Veterinary Medicine, Konkuk University, Seoul 143-701

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Abstract : The number of reporting the effects on ginseng's physiological, pharmacological, and behavioral effects has been increased every year. Major active components of *Panax* ginseng, are the ginsenosides, which are mainly triterpenoid dammarane derivatives. 3-Nitropropionic acid (3-NP) is known to induce cellular energy deficit and oxidative stress related neurotoxicity via an irreversible inhibition of the mitochondrial enzyme succinate dehydrogenase (SDH). Intraperitoneal injection of 3-NP produces striatal degeneration. Aged animals was more vulnerable to 3-NP than young animal. We used three different ages of 5-, 8-, and 26-week-old rats. 3-NP alone treatment induced striatal lesion and increased lesion volume with age-dependent manner in 5-, 8-, and 26-week-old rats by 30.2 ± 5.8, 37.38 ± 6.1, and 51.3 ± 8.4 mm^3, respectively. However, pretreatment of GTS (100 mg/kg/day) before 3-NP reduced striatal lesion in 5-, 8-, and 26-week-old rats by 3.15 ± 6.1, 8.89 ± 1.9, and 27.3 ± 5.6 mm^3, respectively. Pretreatment of GTS also significantly increased survival rate in 5-week-old rats (3-NP alone: GTS + 3-NP = 40.4 ± 6.3: $72.5 \pm 9.5\%$) than 8-week-old rats (3-NP alone: GTS + 3-NP = $13.5 \pm 5.2\%$: $45.1 \pm 3.1\%$). In 26-week-old rats, 3-NP alone treated group died on day 18, whereas GTS + 3-NP-treated group prolonged lifespan to 30 days. Thus, pretreatment of GTS before administration of 3-NP extended lifespan in all ages. The present results indicate that aged animals are more vulnerable to 3-NP and GTS pretreatment protected 3-NP-induced striatal damage in different ages of animals.

Key words : Ginseng saponins; 3-Nitropropionic acid; Striatal degeneration; Aged animal; Neuroprotection

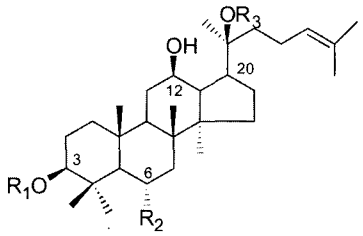
1. INTRODUCTION

Systemic or intraperitoneal administration of 3-nitropropionic acid (3-NP), an irreversible inhibitor of succinate dehydrogenase, elicits striatal neuronal degeneration in rodents and non-human primates, and recapitulates many of the clinical and pathological features of Huntington's disease (HD)[1-3]. 3-Nitropropionic acid (3-NP) is a compound found in crops contaminated with fungi[4] and causes neurotoxicity in both animals and human[5,6]. Since treatment of 3-NP induces a selective striatal pathology similar to that seen in HD, it has been widely used as an agent for animal model study of HD[1,7,8]. The primary mechanism of 3-NP-caused neurotoxicity involves the irreversible inhibition of mitochondrial succinate dehydrogenase (SDH) and leads to inhibition of ATP synthesis[9,10]. ATP exhaustion by mitochondrial dysfunction also subsequently couples to the slow secondary excitotoxicity by excitatory neurotransmitter[11]. This secondary excitotoxicity in ATP deficient neurons is initiated by voltage-dependent Na^+ channel activation, which is coupled to membrane depolarization, Ca^{2+} channel activation, and subsequent NMDA receptor activation by relief of voltage-dependent Mg^{2+} block of the NMDA receptor[12,13]. These serial cascades induced by 3-NP intoxication are also accompanied with the impaired mitochondrial Ca^{2+} homeostasis, with intracellular Ca^{2+} elevation via L-type and other types of Ca^{2+} channel activations, and with an impaired buffering capacity on intracellular Ca^{2+} in astrocytes and neurons[14-17]. Recent *in vivo* studies showed that old animals are more vulnerable to 3-NP than young rats[18].

Ginsenoside total saponin (GTS), which are also known as ginsenosides, are active ingredients isolated from *Panax ginseng* C.A. Meyer, which is a well-known tonic medicine[19]. Recent accumulating evidences have shown that treatment of GTS not only attenuates intracellular

To whom correspondence should be addressed.
(Tel) +82-2-450-4154; (Fax) +82-2-450-3037
(E-mail) synah@konkuk.ac.kr

Ca^{2+} elevation by blocking various types of Ca^{2+} channels like L-, N-, and P/Q-types and depolarization-induced Ca^{2+} influx[20-22] but also inhibits receptor agonist-induced intracellular Ca^{2+} mobilization in neurons[23]. GTS also reduces glutamate/NMDA-mediated Ca^{2+} influx in neurons[24, 25]. Furthermore, recent reports showed that these GTS-mediated inhibitions on intracellular Ca^{2+} elevations could be the basis of *in vitro* or *in vivo* protection against excitatory amino acids- or neurotoxins-caused neuronal cell damages. For example, GTS attenuated glutamate or kainic acid-caused cortical, hippocampal, spinal cord neuron damages in rats[24, 26-28]. GTS also attenuated 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or 1-methyl-4-phenylpyridinium (MPP^+)-induced dopaminergic neuron deaths in rat or dopaminergic cell culture in mice[29, 30]. However, until now little is known about protective effect of GTS following in age-dependent manner against progressive striatal degeneration induced by 3-NP, although we have shown in previous report that GTS protected 3-NP-induced striatal degeneration in rats[31]. We therefore examined whether systemic administration of GTS could exert protective effects against systemic 3-NP- induced rat striatal degeneration in a age-dependent manner. Herein, we present results that 3-NP produced age-dependent lesions in rat striatum and GTS protected the 3-NP-induced striatal degeneration in all ages tested.



Ginsenosides	R_1	R_2	R_3
Rb_1	-Glc_2-Glc	-H	-Glc_6-Glc
Rb_2	-Glc_2-Glc	-H	-Glu_6-Ara(pyr)
Rc	-Glc_2-Glc	-H	-Glc_6-Ara(fur)
Rd	-Glc_2-Glc	-H	-Glc
Re	-H	-O-Glc_2-Rha	-Glc
Rf	-H	-O-Glc_2-Glc	-H
Rg_1	-H	-O-Glc	-Glc
Rg_2	-H	-O-Glc_2-Rha	-H
Rg_3	-Glc_2-Glc	-H	-H

Fig. 1. Structures of the nine representative ginsenosides. They differ at three side chains attached the common steroid ring. Abbreviations for carbohydrates are as follows: Glc, glucopyranoside; Ara (pyr), arabinopyranoside; Rha, rhamnopyranoside. Superscripts indicate the carbon in the glucose ring that links the two carbohydrates.

2. MATERIALS AND METHODS

2.1. Drugs

Fig. 1 shows the structures of the nine representative ginsenosides. GTS was kindly obtained from Korea Ginseng Corporation (Taejon, Korea). GTS contained Rb_1 (17.1%), Rb_2 (9.07%), Rc (9.65%), Rd (8.26%), Re (9%), Rf (3%), Rg_1 (6.4%), Rg_2 (4.2%), Rg_3 (3.8%), Ro (3.8%), Ra (2.91%) and other minor ginsenosides. GTS was diluted with bath medium or saline before use. 3-NP and other chemicals were of analytical grade and purchased from Sigma (St. Louis, MO).

2.2. Drug administrations

Male Sprague-Dawley rats were utilized. Animals were housed in groups of four per cage in a room with controlled temperature and humidity, and on a 12-h light-dark cycle with lights. Food and water were available *ad libitum* throughout the experiments. Their care and handling were in accordance with the highest standards of institutional guidelines. In experiment on 3-NP striatal degeneration, animals were divided into 5-, 8-, and 26-week-old group. Again, each group was divided into control saline, 3-NP alone, and GTS + 3-NP group (n=15, respectively). Also, in study on 3-NP-induced mortality, animals were divided into 5-, 8-, and 26-week-old group (n=20, each group). Control vehicle group was administered only with saline. GTS dissolved in saline was administrated intraperitoneally (i.p.) to rats with dose (50 mg/kg, twice/day 3 h before 3-NP administration, every 12 h interval for 42 days, each group). GTS administration began on day 0, followed by 3-NP (10 mg/kg, i.p., once every 3 days) on day 3. Fresh 3-NP powder was dissolved in saline and the pH was adjusted to 7.4 with NaOH.. After removal brain, striatal lesion volume was determined by TTC staining as described below.

2.3. TTC staining, striatal lesion volume measurement

At the end of drug administration, animals were sacrificed for 2,3,5-triphenyltetrazolium chloride (TTC) staining. Brains were quickly removed and placed in ice-cold saline solution. Brains were sectioned at 2 mm intervals using rat brain matrix. Slices were then subjected in 2% TTC for 5 min at 37°C in the dark and removed and placed in 4% paraformaldehyde, pH 7.4 in 0.1 M phosphate buffer. For measurement of lesion volumes, serial, coronal sections (25 mm) were cut throughout the entire striatum using a cryostat and every fourth section was

thaw-mounted on gelatin-coated slides[32]. Using computer-based image analysis (Image, NIH), lesion volumes of each age-dependent group were calculated by summing the cross-sectional area of the lesion in each section and multiplying this value by the distance between sections. The experiment was accomplished 2 times, repeatedly.

2.4. Survival test

In survival experiments, twenty animals were randomly divided into 5-, 8-, and 26-week-old group. Again, each group was divided into control vehicle, 3-NP alone, or GTS (50 mg/kg, twice/day, i.p.) + 3-NP group (10 mg/kg, once every 3 days, i.p.). Other procedures for drug administrations were same as described above. After drug administrations, twenty animals were randomly divided into control vehicle, 3-NP alone, or GTS (50 mg/kg, twice/day, i.p.) + 3-NP group (n = 20, each group), respectively. Other procedures were same as described above. Animals in each group were observed twice daily, early morning and late afternoon. Some of animals treated with 3-NP begin to show severe limb paralysis, dystonia, or other abnormal behaviors after 3-4 times administration. The criterion for euthanization was the point in time at which animals were unable to initiate normal movement after being gently plodded for 2 min.

2.4.1. Data analysis

Data are expressed as mean ± S.E.M. For statistical comparisons, ANOVA followed by Tukey's test was used for multiple comparisons and Student's t test for pairs of data. $P < 0.05$ was considered significant.

3. RESULTS

3.1. Effect of GTS on 3-NP- induced rat striatal degeneration

We first examined the effects of GTS on neurotoxicity induced by repeated treatment of 3-NP using TTC staining method. The dosage of GTS (50 mg/kg, twice/day 3 h before 3-NP, every 12 h interval) were administered on

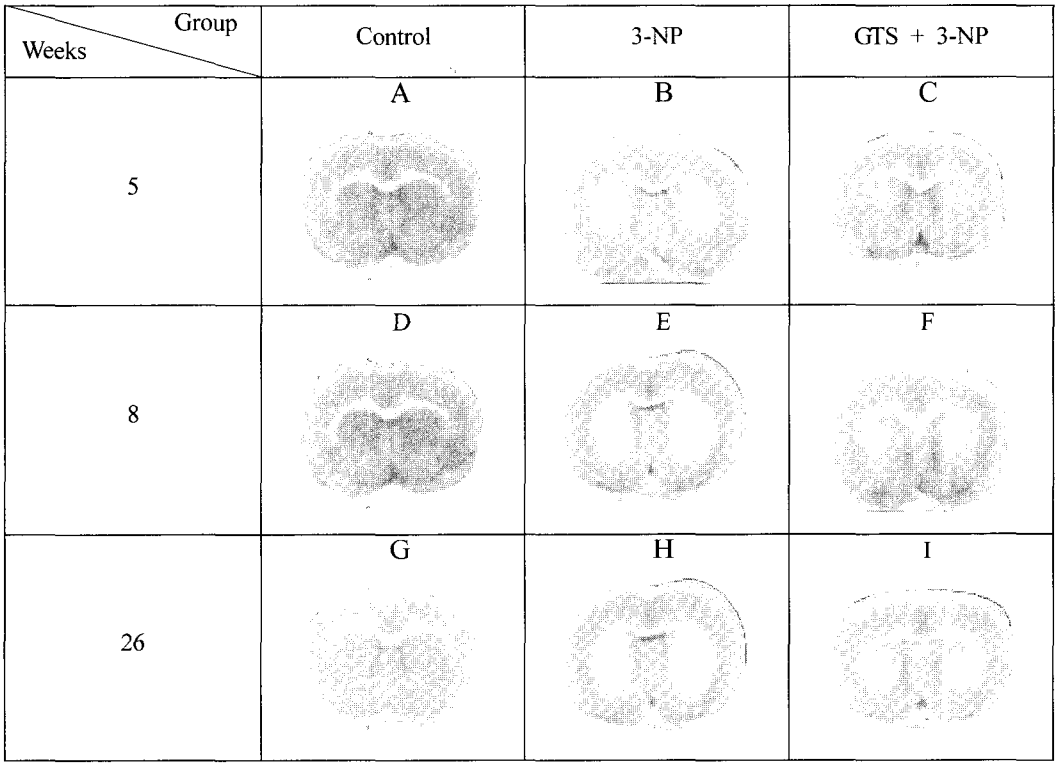


Fig. 2. TTC-stained coronal sections taken from control, 3-NP alone, and GTS+3-NP-injected animals with age-dependent manner. Histologic studies showed a striking age dependence of the lesions, with 5- (A, B and C), 8- (D, E and F), and 26-week-old rats (G, H and I). Pretreatment of ginsenosides (GTS) attenuates 3-NP alone induced-rat striatal degeneration with age-dependent manner. The representative coronal brain sections of saline control (A, D and G), 3-NP alone (B, E and H), or GTS + 3-NP (C, F and I) were shown. The detailed experimental procedures for drug administration and TTC staining were described in Material and Methods section.

day 0, followed by 3-NP (10 mg/kg, i.p., once every 3 days) on day 3 in different ages of rats (5-, 8-, and 26-week-old). On day 42, 3-NP and GTS administration were discontinued. At the end of drug administration, animals were sacrificed for 2,3,5-triphenyltetrazolium chloride (TTC) staining. Fig. 2 shows representative TTC stainings divided into 5- (A, Control; B, 3-NP alone; C, 3-NP + GTS), 8- (D, Control; E, 3-NP alone; F, GTS + 3-NP), and 26-week-old group (G, Control; H, 3-NP alone; I, GTS + 3-NP). In gross TTC staining of coronal brain sections, animals treated with 3-NP alone displayed bilateral striatal lesions but in young age group of rat (5-week-old) 3-NP induced less striatal damage compared to the other groups, supporting that young animals were less sensitive to 3-NP than old animals. Next, we tested whether pretreatment of GTS could protect striatal damage induced by 3-NP in old group of rat. As shown in Fig. 2, the protective effect of GTS against 3-NP-induced striatal degeneration was not dependent on animal age, although the degree of striatal degeneration by 3-NP in rats treated with GTS was increased in 26-week-old rat group. Thus, we could observe significant protective effects of GTS in different age group (5-week-old: 8-week-old : 26-week-old = 89.5 ± 6.1 : 76.4 ± 5.9 : 46.8 ± 8.4%, compared with 3-NP alone treatment, $*P<0.001$, n=15, each group) (Fig. 3A).

3.2. Effect of GTS on 3-NP-induced mortality

The effects of GTS on 3-NP-induced mortality were also investigated. In this experiment, some of animals treated with 3-NP showed severe limb paralysis, dystonia, or other abnormal behaviors after 3-4 times administration. Since these animals themselves lost the ability to eat food and died if they were not paid with further cares, we considered them as dead and have euthanized them as previously reported[33]. The survival rate was significantly correlated with age-dependence. We could observe a long survival rate in 5- and 8-weeks-old rats for 42 days (5-week-old : 8-week-old = 40.4 ± 6.3% : 13.5 ± 5.2% in 3-NP alone group), whereas the survival rate in 26-week-old-rats was strikingly decreased. Thus, all animals treated with 3-NP alone died on day 18. Next, we examined the effect of GTS on 3-NP-induced mortality. Pretreatment of GTS extended the survival rate in all age groups (3-NP alone : GTS + 3-NP = 40.4 ± 6.3% : 72.5 ± 9.5% in 5-week-old; 3-NP alone : GTS + 3-NP = 13.5 ± 5.2% : 45.1 ± 3.1% in 8-week-old). We also could observe extension effect of GTS on survival rate in 26-week-old rats from 18 day to 30 day. These results suggest that GTS prolongs

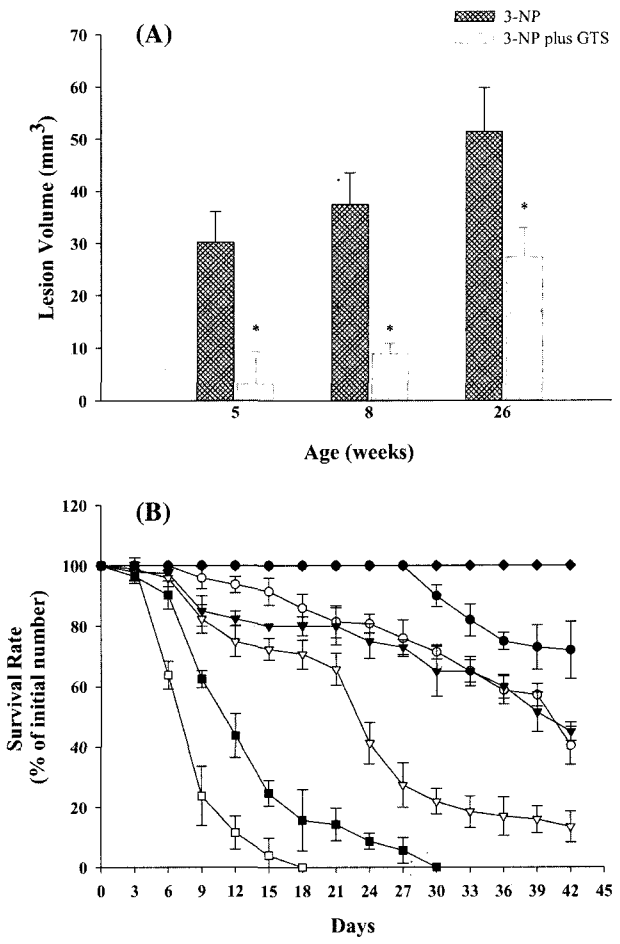


Fig. 3. (A) The summary of percent blockade on 3-NP induced-striatal lesion by GTS with age-dependent manner. A. The histograms show the percent blockade on 3-NP induced-striatal lesion by GTS with age-dependent manner. ($*P < 0.001$, compared with 3-NP alone treatment. n = 15, each group). Data represent the means ± S.E.M.
(B) The effects of GTS on 3-NP-induced mortality was determined. Survival rate of control saline-treated group (◆) were 100% for 42 days in 5-, 8- and 26-week-old group. 3-NP alone-treated rats in all group exhibited wide lesions in striatum (○: 5 weeks-old, ▽: 8 weeks-old, and □: 26 weeks-old). But, GTS plus 3-NP treated-group significantly decreased lesions compared with 3-NP alone group (●: 5 weeks-old , ▼: 8 weeks-old, and ■: 26 weeks-old; n = 20, each group. The mean survival of 5-week-old rats was 72.5% in GTS plus 3-NP group, and was 40.37% in 3-NP alone group after 14 times administration of 3-NP. The mean survival of 8-week-old rats was 45.1% in GTS plus 3-NP group, and was 13.47% in 3-NP alone group after 14 times administration of 3-NP. But, all 26-week-old rat died after only 9 times administration of 3-NP, and died in 3-NP alone group after 5 times administration of 3-NP (n = 20, each group).

life-span of aged animals under influence of 3-NP as observed in attenuation of striatal lesion by GTS (Fig. 3B). The experiment also was accomplished 2 times, repeatedly, and we confirmed a characteristic reappearance.

4. DISCUSSION

Ginsenosides are unique saponins, which only exist in *Panax* ginseng, with pharmacological effects in central and peripheral nervous systems[19]. Recent studies showed that ginsenosides could exert *in vitro* and *in vivo* protective actions against acute excessive stimulation of excitatory neurotransmitters[24, 26, 27, 34]. The present study further extended that GTS could also protect central nervous system from repeated neurotoxic insults. Thus, we demonstrated that intraperitoneal administration of GTS exhibited protective effects against 3-NP-induced age-dependent striatal lesions. We could also observe that GTS showed significant beneficial effects by reducing 3-NP-caused age-dependent mortality.

One of the main indicators of neuronal excitotoxicity or excitotoxin-induced cell death is derived from the disturbance of intracellular Ca^{2+} homeostasis. Recent reports showed that 3-NP-caused cellular ATP exhaustion is coupled to intracellular Ca^{2+} elevation. 3-NP-induced intracellular Ca^{2+} elevation might be mediated via at least two pathways; first is through voltage dependent Ca^{2+} channel following depolarization with Na^{+} influx, second is through NMDA and non-NMDA receptor activation[14, 15, 35]. What is the mechanism underlying the protective effect of GTS against 3-NP-caused rat striatal neurotoxicity? One possibility is that GTS-induced protection against 3-NP neurotoxicity might be derived from the inhibition on 3-NP-induced Ca^{2+} influx via L- and other types of Ca^{2+} channel. Previous report showed that 3-NP-induced intracellular Ca^{2+} elevation was mediated via a L-type and other types of Ca^{2+} channels[14]. We have shown that ginsenosides inhibit L-, N-, and P/Q-types of Ca^{2+} channels[20-22, 36]. The second possibility might be derived from ginsenoside-induced attenuation of extracellular Ca^{2+} entry caused by NMDA receptor activation, which might be secondarily induced by 3-NP intoxication[11, 35]. In previous studies, our group showed that ginsenosides not only inhibit NMDA receptor-mediated current and -Ca^{2+} influx but also attenuate kainate-induced hippocampal neuron deaths[25, 27]. Thus, these GTS-induced limiting actions on extracellular 3-NP-induced Ca^{2+} influx via Ca^{2+} channels and subsequent Ca^{2+} influx via secondarily NMDA receptor activation might help not to aggravate 3-NP-induced intracellular Ca^{2+} unbalance. Moreover, these contributions of GTS might help to diminish ATP consumption needed for maintaining intracellular ionic balances in striatal cells under 3-NP or malonate insults and finally ameliorate 3-NP- or malonate-induced neurotoxicity. Similarly, MK801, a NMDA receptor antagonist, or riluzole, which inhibits neuronal voltage-dependent Ca^{2+} and Na^{+} channel activity, not only attenuated 3-NP-induced Ca^{2+} elevation but also exhibited neuroprotective effects against 3-NP-induced neurotoxicity[35, 37]. The last possibility is that GTS-induced neuroprotection against 3-NP neurotoxicity might be derived from the attenuation of oxidative stress caused by glutamate in striatal cells under 3-NP insults, since ginsenosides inhibit glutamate mediated-overproduction of NO and malonyldialdehyde and prevented a decrease of superoxide dismutase activity in glutamate-treated cortical neurons[21, 26, 34]. However, it is unlikely that the protective effects of GTS are due to direct interaction with 3-NP, activation of SDH, or blocking action of SDH inhibitor, since in previous studies, GTS itself had no effect on SDH activity and GTS administered 3 h before 3-NP treatment also did not affect 3-NP-induced inhibition of SDH activity[35]. It is also possible to say that GTS treatment may accelerate the elimination of 3-NP, rendering the neurotoxin less active. Here, this possibility can be clearly ruled out, since GTS is active *in vitro* where 3-NP concentrations remain stable. Taken together, the main contributing factors on GTS-induced protection against 3-NP neurotoxicity could be due to the inhibitory effects on intracellular Ca^{2+} elevations, that might be due to Ca^{2+} channel and NMDA receptor activations.

In summary, using a rodent HD model system that shows a selective striatal lesion by 3-NP treatment with age-dependent manner, we obtained results suggesting that GTS has neuroprotective effects against 3-NP-induced age-dependent striatal neurotoxicity.

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