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Research article

Proteomic change by Korean Red Ginseng in the substantia nigra of a Parkinson's disease mouse model

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ABSTRACT

Background: Recent studies have shown that Korean Red Ginseng (KRG) successfully protects against dopaminergic neuronal death in the nigrostriatal pathway of a Parkinson's disease (PD) mouse model induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration; however, the mechanism has yet to be identified. Therefore, in this study we used two-dimensional electrophoresis to investigate the effects of KRG on the changes in protein expression in the substantia nigra (SN) of MPTP-treated mice.

Methods: Male C57BL/6 mice (9 wk old) were intraperitoneally administered MPTP (20 mg/kg) four times at 2-h intervals, after which KRG (100 mg/kg) was orally administered once a day for 5 d. Two hours after the fifth KRG administration, a pole test was conducted to evaluate motor function, after which the brains were immediately collected. Survival of dopaminergic neurons was measured by immunohistochemistry, and protein expression was measured by two-dimensional electrophoresis and Western blotting.

Results: KRG alleviated MPTP-induced behavioral dysfunction and neuronal toxicity in the SN. Additionally, the expression of eight proteins related to neuronal formation and energy metabolism for survival were shown to have changed significantly in response to MPTP treatment or KRG administration. KRG alleviated the downregulated protein expression following MPTP administration, indicating that it may enhance neuronal development and survival in the SN of MPTP-treated mice.

Conclusion: These findings indicate that KRG may have therapeutic potential for the treatment of patients with PD.

1. Introduction

Parkinson's disease (PD) is common among the elderly, and its prevalence increases from 1% in people older than 60 years to 4% in those older than 80 years [1]. PD is a chronic progressive neurodegenerative disorder characterized by a selective loss of nigrostriatal dopaminergic neurotransmission caused by neuronal cell death in the substantia nigra (SN) [2,3]. The administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin that induces selective damage to dopaminergic neurons in the SN, causes behavioral impairments similar to those seen in PD patients. Accordingly, the MPTP-treated animal model is well known and commonly used to reproduce neurodegenerative conditions mimicking PD [4].

Panax ginseng Meyer (ginseng) is a beneficial herb used as a crude material to treat inflammation and enhance energy and immunity in Asian countries. The pharmacological and clinical beneficial effects of ginseng have been shown to include anticancer, antiaging, and anti-inflammation activities via promoting human immune response [5]. Korean Red Ginseng (KRG) is steamed and dried for lengthy preservation, which results in conversion of some of the chemical profiles of ginsenosides [6]. Previous studies have

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shown that ginseng and KRG suppressed 1-methyl-4-phenylpyridinium-induced cell death in PC12 [7,8] and SH-SY5Y [9] cells, and that oral administration of KRG extract protected dopaminergic cell death in the nigrostriatal pathway from MPTP toxicity [10,11] and improved cognitive functions in patients with Alzheimer's disease [12]. Moreover, ginsenoside Rg1 attenuated MPTP-induced behavioral impairment and dopaminergic neuronal death through the Wnt/β-catenin signaling pathway [13] and by reducing neuroinflammation [14], whereas the ginsenoside Rd prevented 1-methyl-4-phenylpyridium-induced cell death in SH-SY5Y cells [15], and ginsenoside Rb1 suppressed α-synuclein aggregation and toxicity [16], indicating that KRG has the potential to alleviate PD symptoms; however, the mechanism is not fully understood.

A previous study showed that KRG prevented MPTP-induced proteomic changes in the striatum, but it is still not clear whether KRG can alleviate the proteomic changes in the SN. Investigating proteomic changes in the SN is important to understanding the mechanism of KRG on PD because PD is characterized by dopaminergic neuronal death in the SN [1]. Two-dimensional gel electrophoresis (2-DE), an analytical method for separating and distinguishing complex protein mixtures [17], is a useful method for identifying changes in the expression of proteins in tissues [18]. In the present study, we used 2-DE to investigate the changes in protein expressions in response to KRG administration in the SN of MPTP-treated mice.

2. Materials and methods

2.1. Animals and groups

This study was approved by the Pusan National University Institutional Animal Care and Use Committee, Yangsan, Korea. Male 9-wk-old C57BL/6 mice (Orientbio Inc., Seongnam, Korea) weighing 20–23 g were housed at room temperature (22 ± 2°C) under a standard 12-h light/dark cycle with unlimited access to food and water. The animals were handled in accordance with the current guidelines established in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1985).

Mice were randomly assigned to three groups: saline-injected group (saline group), MPTP-injected group (MPTP group), or MPTP-injected plus 100 mg/kg KRG-treated group (KRG group).

2.2. MPTP injection and KRG administration

All mice except those in the saline group were injected with MPTP–HCl (20 mg/kg; Sigma, St. Louis, MO, USA) intraperitoneally four times at 2-h intervals (total, 80 mg/kg) [19]. Mice in the saline group were injected with vehicle (normal saline) on the same schedule.

The KRG extract used in this study was obtained from Korea Ginseng Corporation (Daejeon, Korea). Briefly, ginseng was steamed at 90–100°C under no pressure for 3 h, dried at 50–80°C, and then extracted three times with circulating hot water at 85–90°C for 8 h each. The water content of the pooled extract was 36% of the total weight. Analysis of the content of crude ginsenoside in the extract by HPLC revealed that it contained 6.92 mg/g Rb1, 2.68 mg/g Rb2, 3.24 mg/g Rc, 0.94 mg/g Rd, 1.40 mg/g Re, 1.03 mg/g Rf, 1.12 mg/g Rg1, 1.23 mg/g Rg2s, 1.03 mg/g Rg3r, 1.98 mg/g Rg3s, 0.89 mg/g Rh1, and other minor ginsenosides.

The KRG extract was diluted with sterilized mineral water to the appropriate concentrations. One hour after the first MPTP injections, mice in the MPTP + KRG group were orally administered the KRG extract (100 mg/kg) at 24-h intervals for 5 consecutive d. Mice in other groups were administered the same amount of mineral water on the same schedule [10].

2.3. Pole test

Mice ($n = 6$ in each group) were mounted head-downward near the top of a rough-surfaced wood pole (10 mm in diameter and 55 cm in height), and the time taken to reach the bottom of the pole was measured [20]. The test was repeated three times at 30-s intervals, after which behavioral changes were evaluated according to the average of the three times. The test was performed 1 d prior to MPTP injection (Day 0) and 2 h after the last oral administration of KRG (Day 5).

2.4. Immunohistochemistry

Following the last pole test, mice were perfused with 4% paraformaldehyde dissolved in 0.1M phosphate buffer (PFA), after which the brain was quickly harvested, postfixed in 4% PFA for 48 h, and immersed in 30% sucrose solution for storage at 4°C prior to sectioning. Frozen sections were then cut to a thickness of 35 μm using a cryostat Leica CM3050S (Leica Microsystems, Wetzlar, Germany). The SN sections located between anterior-posterior −3.08 and −3.28 mm from the bregma were incubated with 1% H_2O_2 in 0.05M phosphate-buffered saline for 15 min, followed by 0.3% Triton X-100 and 3% normal blocking serum in phosphate-buffered saline at room temperature for 1 h, after which they were stained overnight at room temperature using an antityrosine hydroxylase (TH, 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibody. The next day, sections were incubated with Vectastain Elite ABC reagents (Vector Laboratories Inc., Burlingame, CA, USA) at room temperature for 1 h, then incubated with a diaminobenzidine substrate kit (Vector Laboratories Inc.) for 5 min. The tissues were subsequently mounted on gelatin-coated slides, air-dried, dehydrated, and coverslipped. Histological pictures were taken using an Axio Scope A1 microscope (Zeiss, Oberkochen, Germany) and an AxioCam ICc3 camera (Zeiss). The survival of dopaminergic neurons was evaluated by the number of TH-positive neuronal cells in the SN. An independent observer who did not know the expected results manually counted the TH-positive neurons bilaterally in five continuous SN sections, and the cell counts were confirmed three times.

2.5. Two-dimensional protein electrophoresis

The tissues of SN were harvested ($n = 3$ at each group) and kept at −80°C until use. Next, tissues were homogenized using a motor-driven homogenizer (PowerGen125; Fisher Scientific, Pittsburgh, PA, USA) in sample lysis solution composed of 7M urea and 2M thiourea containing 4% (w/v) 3-[(3-cholamidopropy)dimethyammonio]-1-propanesulfonate (CHAPS), 1% (w/v) dithiothreitol (DTT) and 2% (v/v) pharmalyte, and 1mM benzamidine. After centrifugation at 15,000*g* for 1 h at 15°C, insoluble material was discarded and the soluble fraction was used for 2-DE.

Immobilized pH gradient dry strips (4–10 NL IPG, 24 cm; Genomine, Inc., Pohang, Korea) were equilibrated for 12 h with 7M urea, 2M thiourea containing 2% CHAPS, 1% DTT, and 1% pharmalyte, after which they were loaded with 300 μg of the sample. Isoelectric focusing was then performed at 20°C using a Multiphor II electrophoresis unit (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's protocols. Briefly, the voltage was linearly increased from 150 V to 3,500 V over 3 h for sample entry, then maintained at 3,500 V for 96 kV h until focusing was complete. Prior to the second dimension, strips were incubated in equilibration buffer [50mM Tris-Cl, pH 6.8 containing 6M urea, 2% sodium

dodecyl sufate (SDS), and 30% glycerol] for 10 min, first with 1% DTT and then with 2.5% iodoacetamide. Equilibrated strips were inserted onto SDS-polyacrylamide gel electrophoresis (20 cm × 24 cm, 10–16%), then run at 20°C for 1,700 V h using a Hoefer DALT 2D system (Amersham Biosciences), after which they were silver stained [21].

Quantitative analysis of digitized images was conducted using the PDQuest software (version 7.0; Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The quantity of each spot was normalized based on the total valid spot intensity, and the spots that showed a change of more than 1.5-fold in expression compared with the control or normal sample were selected. Clustering analysis was then performed on a correlation matrix for both mice and proteins.

2.6. Protein identification

For protein identification by peptide mass fingerprinting (PMF), protein spots were excised, digested with trypsin (Promega, Madison, WI, USA), mixed with α cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid, and subjected to matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis (Microflex LRF 20; Bruker Daltonics, Billerica, MA, USA). Spectra were collected from 300 shots per spectrum over an m/z range of 600–3,000 and calibrated by two-point internal calibration using Trypsin autodigestion peaks (m/z 842.5099, 2,211.1046). A peak list was generated using Flex Analysis 3.0 (Bruker Daltonik, Bremen, Germany). The threshold used for peak-picking was as follows: 500 for minimum resolution of monoisotopic mass, 5 for S/N. The MASCOT search program (http://www.matrixscience.com/) was used for protein identification by PMF. The following parameters were used for the database search: trypsin as the cleaving enzyme, a maximum of one missed cleavage, iodoacetamide (Cys) as a complete modification, oxidation (Met) as a partial modification, monoisotopic masses, and a mass tolerance of ±0.1 Da. PMF acceptance criteria was based on probability scoring.

2.7. Western blotting

Equal amounts of protein (40 μg) from each sample were separated on 10% SDS-polyacrylamide gels, then transferred to nitrocellulose membranes. The membrane was subsequently blocked with 5% bovine serum albumin in tris-buffered saline containing 0.1% Tween-20 for 1 h at room temperature, after which it was incubated overnight at 4°C with TH (1:2,000; Santa Cruz Biotechnology), antidystrophin-related protein 2 (DRP2, 1:200; Santa Cruz Biotechnology), or anticollapsin response mediator protein 5 (CRMP5, 1:5,000; Thermo Scientific, Rockford, IL, USA) primary antibodies. Next, the membrane was incubated with secondary antibody at room temperature for 1 h. Bands were subsequently detected using an enhanced chemiluminescence detection kit (Thermo Scientific), after which these blots were reprobed with rabbit monoclonal anti-β-actin antibody (1:1,000; Santa Cruz Biotechnology) as a loading control for all experiments. Quantification of immunoreactivity corresponding to the total bands was performed by densitometric analysis using an Image Quant LAS 4000 (Fujifilm, Tokyo, Japan). Western blotting analysis was performed in triplicate.

2.8. Statistical analysis

All data were expressed as the means ± standard deviation. Groups were compared by one-way analysis of variance with the Neuman–Keuls *post hoc* test. All statistical testing was performed using Prism 5 for Windows (GraphPad Software Inc., La Jolla, CA, USA), and statistical significance was set at $p < 0.05$.

3. Results

3.1. KRG recovers abnormal motor function induced by MPTP treatment

There was no significant difference in the duration of descending time among all groups on Day 0. However, the duration was significantly elevated in the MPTP group (16.33 ± 5.65 s, $p < 0.05$) relative to the saline group (10.71 ± 2.10 s) on Day 5. The KRG group showed a significantly decreased duration compared with the saline group (8.44 ± 2.49 s, $p < 0.01$; Fig. 1).

3.2. KRG shows neuroprotective effects against MPTP-induced dopaminergic neuronal loss in the SN

The number of TH-positive neurons in the MPTP group (64.12 ± 25.89%) was significantly decreased relative to that in the saline group (100.0 ± 22.43%, $p < 0.05$). However, the number in the KRG group (91.91 ± 20.80%, $p < 0.05$) was significantly increased relative to that in the MPTP group (Fig. 2).

3.3. KRG modulates MPTP-changed protein expression in the SN

Patterns of protein expression were observed in each group on approximately 691 spots by 2-DE (Fig. 3A). Among these spots, 15 that showed changes in expression of more than 1.5-fold were selected in the pH range of 4–10. The selected protein spots were downregulated in the MPTP group, whereas oral administration of KRG reversed the downregulated protein expression induced by MPTP treatment.

3.4. KRG relieved differentially expressed proteins induced by MPTP treatment

MALDI-TOF analysis led to successful identification of 12 spots based on comparison with the National Center for Biotechnology Information nonredundant database (Fig. 3B and Table 1). Among them, eight were significantly differentially expressed. The spots were identified as DRP2, voltage-dependent anion-selective channel protein 2 (VDAC2), cytosolic acyl coenzyme A thioester hydrolase isoform X3 (ACOT7), 4-aminobutyrate aminotransferase, mitochondrial isoform 1 precursor (ABAT1), 4-aminobutyrate

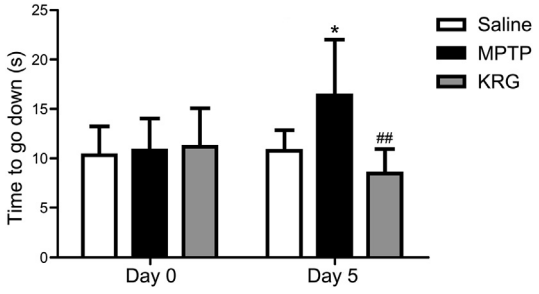

Fig. 1. Effects of Korean Red Ginseng (KRG) on abnormal motor function induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment. MPTP-treated mice required more time to descend than saline-treated mice, but this was significantly restored by oral administration of KRG. Data are presented as means ± standard deviation ($n = 6$ in each group). All results were determined based on one-way analysis of variance (ANOVA) with the Neuman–Keuls *post hoc* test. $^{*}p < 0.05$ compared with the saline group. $^{\#\#}p < 0.01$ compared with the MPTP group. KRG, MPTP-treated and KRG-administered group; MPTP, MPTP-treated group; saline, saline (vehicle)-treated group.

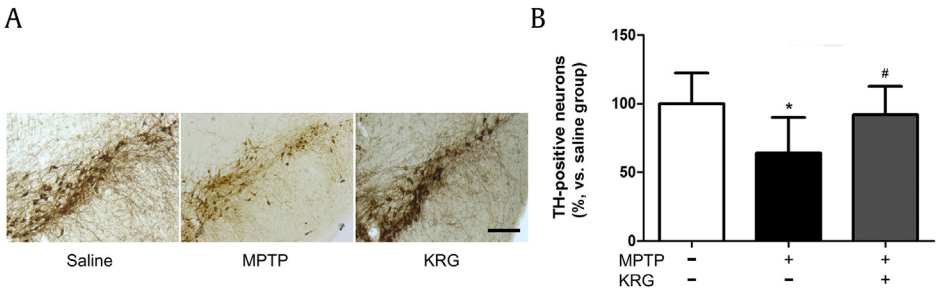

Fig. 2. Effects of Korean Red Ginseng (KRG) on expression of tyrosine hydroxylase (TH)-positive neurons in the substantia nigra (SN). (A) TH-specific immunohistochemical staining in the SN. (B) The number of TH-positive neurons in the SN. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment significantly decreased TH-positive neurons in the SN, whereas KRG administration preserved the MPTP-induced neuronal loss. Scale bar, 200 μm. Data are presented as means ± standard deviation ($n = 6$ in each group). All results were determined based on one-way analysis of variance with the Neuman–Keuls *post hoc* test. $^{*}p < 0.05$ compared with the saline group. $^{\#}p < 0.05$ compared with the MPTP group.

aminotransferase, mitochondrial isoform 2 precursor (ABAT2), CRMP5, pyruvate kinase M (PKM), and T-complex protein 1 subunit eta (TCP1). MPTP treatment downregulated the expression of these proteins. KRG oral administration significantly alleviated the expression of VDAC2, ACOT7, ABAT1, and CRMP5, and the expression of DRP2, ABAT2, PKM, and TCP1 tended to decrease, although not at a significant level (Fig. 4).

3.5. Confirmation of protein changes

To verify the reliabilities of TH immunohistochemistry and proteomic analysis, DRP2 and CRMP5 were selected as representative proteins of 2-DE because they are associated with motor dysfunction and neuronal formation, after which Western blotting was performed. The expression of TH, DRP2, and CRMP5 was consistent with the results of immunohistochemistry and 2-DE analyses (Fig. 5).

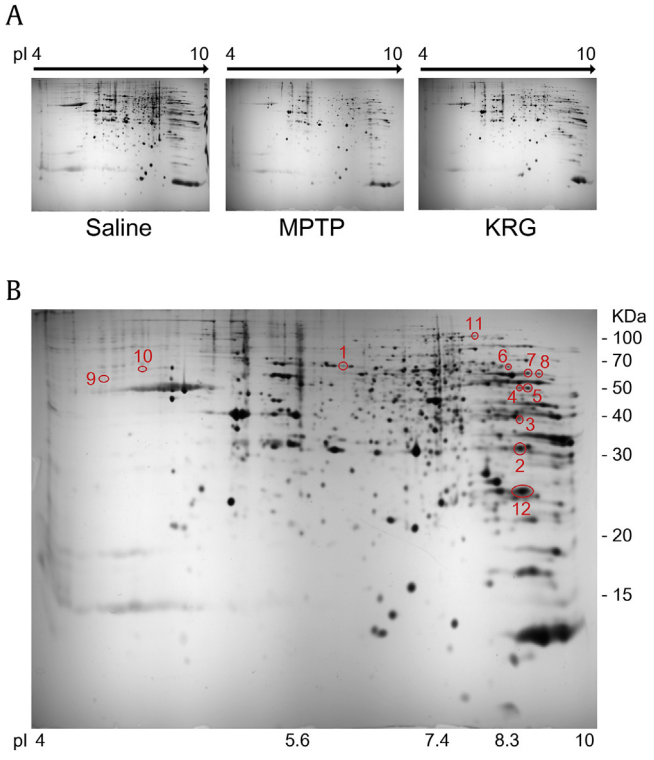

Fig. 3. Protein expressions in the substantia nigra identified by two-dimensional gel electrophoresis. (A) Protein expressions in the substantia nigra obtained over different p*I* ranges. (B) Representation of differentially expressed protein spots. Twelve proteins were identified by MALDI-TOF analysis. KRG, Korean Red Ginseng; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

4. Discussion

The results of the present study demonstrated that KRG protected against MPTP-induced degeneration of dopaminergic neurons in the SN, and that the protein profiles among the saline, MPTP, and KRG groups were differentially expressed based on a comparative proteomic analysis by 2-DE and MALDI-TOF MS. The main findings of the present study were that KRG recovered downregulated expression of proteins closely related to neurodegenerative processes in this region. This neuronal-preserving effect of KRG may indicate the clinical potential for treatment of PD patients. The issues regarding the KRG-modulated proteins are discussed below.

A total of 12 proteins were identified as downregulated in the SN in the MPTP group, and the MPTP-induced changes were recovered by KRG administration. Among them, eight (DRP2, VDAC2, ACOT7, ABAT1, ABAT2, CRMP5, PKM, and TCP1) were significantly altered in response to MPTP, whereas KRG resulted in significantly increased levels of four proteins (VDAC2, ACOT7, ABAT1, and CRMP5) relative to the saline group.

DRP2 plays a critical role in maintenance of the central nerve membrane and is highly enriched in the postsynaptic density organizing central cholinergic synapses [22]. As a result, cognitive impairments have been found in the dystrophin deficiency animal model (mdx mice) [23]. Dystrophin is also localized at the postsynaptic membrane of the gamma-aminobutyric acid (GABA)-ergic synapse; therefore, mdx mice have been reported to show reduced inhibitory GABAergic functions in amygdala, which results in defensive behavior [24]. The SN also acts a major source of GABAergic inhibition; therefore, we can infer that dystrophin deficiency in this region might, in part, develop movement dysregulation.

In this study, the results of DRP2 expression differed slightly from those of Western blot analysis. DRP2 expression in the KRG group was not changed significantly compared with that in the MPTP group when we analyzed 2-DE data. However, KRG was found to upregulate DRP2 expression significantly on Western blotting analysis. This discrepancy may have been attributable to the number of mice ($n = 3$ at each group) used for 2-DE analysis. Actually, KRG administration upregulated DRP2 expression by > 200 times in the 2-DE results, and we conducted Western blot analysis in triplicate, which likely explains the significant difference between MPTP and KRG groups observed in the Western blotting results.

CRMP5 belongs to the CRMP family, which is thought to be involved in neurodevelopmental function through roles in axon specification, elongation, and navigation [25]. Moreover, CRMPs are

Table 1
Differential protein expression profiles of the 12 spots after MPTP treatment and KRG oral administration

Spot No.	Protein name	Protein ID	Theoretical		Gel		Score	Sequence coverage (%)	No. of matched peptides	Fold change	
			M_r (kDa)	pI	M_r (kDa)	pI				Saline/MPTP	KRG/MPTP
1	Dihydropyrimidinase-related protein 2	XP_006518571	62.6	6.0	64.1	6.0	233	44	24	490.10	234.98
2	Voltage-dependent anion-selective channel protein 2	NP_035825	32.3	7.4	30.8	8.4	159	60	14	2.35	2.04
3	Cytosolic acyl coenzyme A thioester hydrolase isoform X3	XP_006539231	36.4	7.2	38.2	8.4	126	47	17	2.05	1.92
4	4-Aminobutyrate aminotransferase, mitochondrial isoform 1 precursor	NP_766549	57.1	8.4	49.5	8.4	274	51	28	553.82	395.48
5	4-Aminobutyrate aminotransferase, mitochondrial isoform 2 precursor	NP_001164449	50.8	8.8	49.5	8.6	187	45	21	4.07	2.97
6	Collapsin response mediator protein 5	NP_075534	62.1	6.6	63.6	8.2	341	54	30	2.44	2.78
7	Pyruvate kinase M	BAA07457	58.4	7.6	58.9	8.6	142	49	22	4.44	1.75
8	T-complex protein 1 subunit eta	NP_031664	60.1	8.0	58.5	8.9	133	29	17	3.22	2.00
9	M2-type pyruvate kinase	CAA65761	58.5	7.2	54.5	4.3	224	50	23	200.38	358.44
10	Syntaxin binding protein 1	AF326545_1	67.9	6.5	61.7	4.6	251	42	25	414.27	379.79
11	Dynamin-1 isoform X7	XP_006497718	97.8	6.7	101.5	7.7	227	36	32	2.34	1.84
12	TPI	AAC36016	27.0	6.9	24.5	8.4	173	67	16	1.96	1.66

KRG, Korean Red Ginseng; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

cytosolic proteins that are highly expressed during intracellular neural development of the brain [26]. In CRMP5−/− mice, brain-derived neurotrophic factor-induced dendritic branching is decreased, and axonal atrophy and motor dysfunction are developed [27]. Although the relationship between CRMP5 deficiency in the SN and PD symptoms is unclear, we can speculate that the lack of CRMP5 might be related to the reduced neuronal outgrowth.

VDAC2 is a protein that arbitrates the flow of energetic metabolites and ions into the outer mitochondrial membrane transport system and is known to inhibit mitochondrial apoptosis by recruiting Bcl-2 homologous antagonist killer (BAK) to the mitochondrial membrane as an inactive conformer, whereas VDAC2 deficiency promotes BAK oligomerization, which increases the susceptibility to apoptotic cell death [28]. VDAC2 expression was downregulated in the adult rat hippocampus following prenatal stress [29], which indicates that VDAC2 expression in the brain region may be suppressed by stress conditions that can occur during brain neuronal apoptosis.

ACOT7 belongs to the type II acyl-coenzyme A thioesterase family and is encoded by the *Acot7* gene in humans. This enzyme is involved in the catalysis of the thioester bonds of activated fatty acids, which leads to the release of nonesterified fatty acid and coenzyme A [30]. The *ACOT7* gene is highly expressed in the brain and the testis of mice [31], and ACOT7 conditional knockout ($Acot7^{N-/-}$) mice showed symptoms of failure in adaptive energy metabolism, which is common in neurodegenerative diseases [32].

ABAT, which is involved in the catalysis of GABA into succinic semialdehyde, is encoded by the *ABAT* gene. There have been case

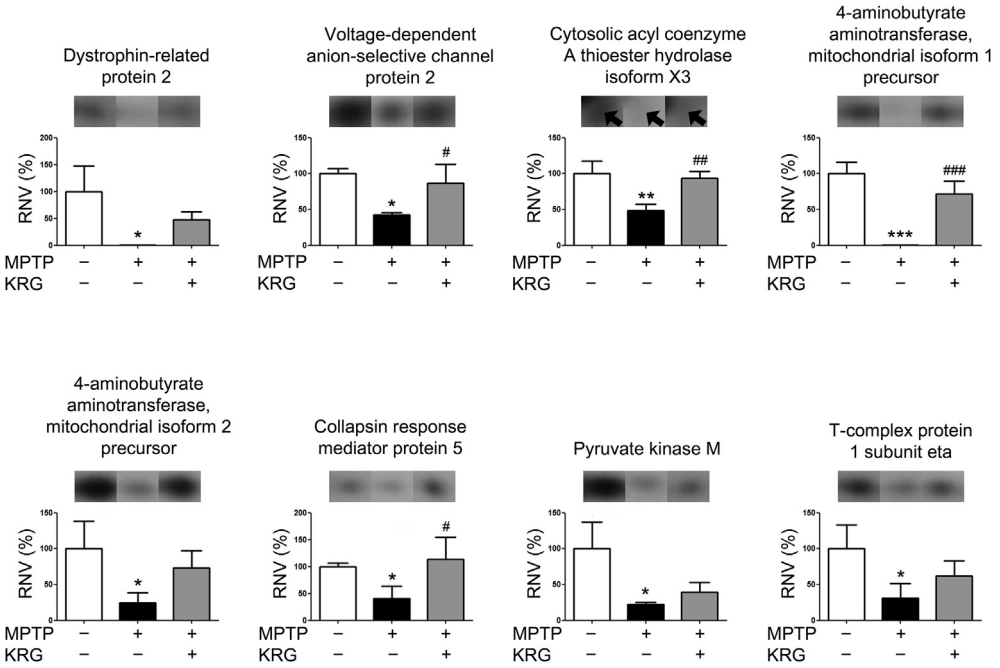

Fig. 4. Effects of Korean Red Ginseng (KRG) on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced differential profiles of protein expressions in the substantia nigra. The levels were illustrated as the relative normalized volumes (RNV) of the proteins. Data are shown as the means ± standard deviation ($n = 3$ in each group). All results were determined based on one-way analysis of variance with the Neuman–Keuls *post hoc* test. Arrowheads represent protein spots. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the saline group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.01$ compared with the MPTP group.

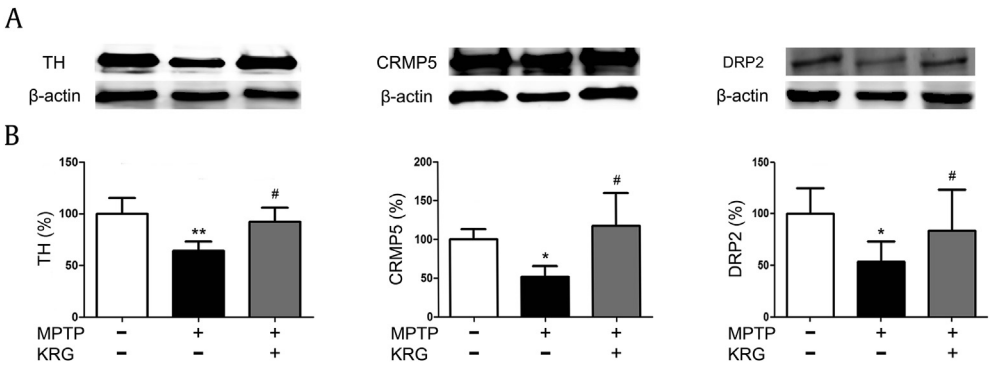

Fig. 5. Verification of immunostaining and proteomic analysis using Western blotting. (A) Changes in the protein expressions of tyrosine hydroxylase (TH), dihydropyrimidinase-related protein 2 (DRP2) and collapsin response mediator protein 5 (CRMP5). (B) Relative values of Cdk5, p35, and p25 expressions. TH expression in the substantia nigra showed a similar trend as immunostaining, and the DRP2 and CRMP5 showed a similar trend as the results from 2-DE analysis. Data are shown as the means ± standard deviation ($n = 3$ in each group), and all results were determined based on one-way ANOVA with the Neuman–Keuls *post hoc* test.

reports regarding ABAT deficiency in which the patients showed increased GABA levels in the plasma and cerebrospinal fluid and associated symptoms such as encephalopathy, psychomotor retardation, seizures, hypotonia, hyperreflexia, lethargy, and abnormal electroencephalogram [33,34].

Pyruvate kinase is an enzyme involved in the final step of glycolysis; therefore, it plays an important role in energy-generating pathways. PKM1 is a PKM isozyme that primarily exists in the brain [35]. Because the brain requires high energy for functioning and survival, PKM deficiency may affect its vulnerability to neuronal damage [36]. MPTP treatment reduces the expression of the specific subunit of system xc- in the SN [37], and the subsequently increased extracellular cysteine may inhibit pyruvate kinase activity [38]. Therefore, we can speculate that the reduced PKM expression in response to MPTP may be mediated by the high concentration of extracellular cystine in this region.

TCP1 is encoded by the *CCT7* gene in humans and plays a crucial role in the formation of molecular chaperone of TCP1 complex, which promotes proper maturation and expression of G protein-coupled receptors and regulates their aggregation [39]. TCP1 expression in MES23.5 cells was decreased by extracellular α-synuclein treatment, which has been considered the cause of PD [40].

When we classified the eight proteins by function, they were found to be associated with the pathophysiology of neurodegenerative diseases and neuronal survival, and VDAC2, ACOT7, and PKM were related to energy metabolism in cells. Interestingly, changed proteins in the previous study were associated with energy metabolism, mitochondrial respiratory chain, and neuroprotection [11]. These results indicate that KRG can suppress MPTP-induced neuronal death and metabolic impairment by regulating the proteins.

In summary, MPTP treatment decreased the expression of proteins related to neuronal formation and energy metabolism for survival, and KRG administration successfully protected against MPTP-induced neuronal toxicity. We also reconfirmed that CRMP5 and DRP2 were downregulated by MPTP treatment, and that KRG administration was reversed through Western blot methodology, which indicates that the changes in the profile of proteins following MPTP treatment and KRG administration observed in the present study are reliable.

The results of the present study indicated that KRG fortified the MPTP-decreased protein expression involved in pathways of neuronal development and energy metabolism; however, it should be noted that there were several limitations that should be addressed in future studies. First, we only observed early-stage proteomic changes in response to MPTP treatment and KRG administration; therefore, the investigation of proteomic changes in the later stages is needed. Second, we did not determine how the changed profile of protein expression affects dopaminergic neuron survival in the region limited to the SN. Third, we did not determine how changes in the manufacturing process profile of ginsenosides contribute to the neuroprotective effects of KRG. KRG contains more various contents of ginsenosides by converting reactions during the manufacturing process [6]. Specifically, ginsenosides Rb1, Rg1, Rd, and Re are considered the major compounds, whereas notoginsenoside R2 and pseudoginsenoside-F11 are the minor compounds responsible for the effectiveness of ginsenosides against PD [41]. Among them, ginsenoside Rg1's modulating Wnt/β-catenin signaling [13] and reducing neuroinflammation [14] may contribute to KRG's neuroprotective effect; however, additional action potentials of other ginsenosides remain to be elucidated. Overall, the results of the present study indicate that KRG has therapeutic potential for the treatment of PD, but that further investigation of the ginsenoside profile may provide more valuable information.

5. Conclusion

KRG reversed MPTP-induced motor disturbance and dopamine neuronal toxicity in the SN and restored MPTP downregulated expression of proteins related to neuroprotection and energy metabolism. Overall, the results of the present study indicate that KRG might have the potential for treatment of PD patients, and that the neuroprotective effects of KRG could, in part, originate from the various contents of ginsenosides.

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Conflicts of interest

The authors declare that no competing financial interests or conflicts of interest with this study exist. The funders had no personal relationships or affiliations with the authors and played no role in the study design, information collection and data analysis, or decision to publish the manuscript.

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