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Ukgansan protects dopaminergic neurons from 6-hydroxydopamine neurotoxicity via activation of the nuclear factor (erythroid-derived 2)-like 2 factor signaling pathway

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ABSTRACT

The sustenance of redox homeostasis in brain is the crucial factor to treat Parkinson's disease (PD). Nuclear factor (erythroid-derived 2)-like 2 factor (Nrf2)-mediated antioxidant response is well known for the main cellular endogenous defense mechanisms against oxidative stress. This study investigated for the first time the effects and possible mechanisms of action of Ukgansan on 6-hydroxydopamine (6-OHDA)-induced neurotoxicity in both *in vitro* and *in vivo* models of PD. We investigated the protective effect of Ukgansan against 6-OHDA with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. In addition, we demonstrated that Ukgansan significantly increased the expression of antioxidant response elements (ARE) and pro-survival protein as Bcl2 and suppressed the expression of pro-apoptotic factors, such as Bax, cytochrome *c*, and caspase-3 using immunoblotting. For the *in vivo* study, we used a mouse model of PD involving stereotaxic injection of 6-OHDA into the striatum (ST). Ukgansan alleviated motor dysfunctions induced by 6-OHDA followed by pole, open-field, and rotation tests. Dopaminergic neuronal loss and Nrf2 activation were evaluated by immunohistochemistry in the mouse ST and substantia nigra pars compacta (SNpc) regions. Ukgansan significantly protected dopaminergic neurons from 6-OHDA toxicity in mouse ST and SNpc by activating Nrf2. These results indicate that Ukgansan inhibited 6-OHDA-induced dopaminergic neuronal cell damage via activation of Nrf2 and its related factors in 6-OHDA-induced dopaminergic loss *in vitro* and *in vivo*. Thus, Ukgansan might delay the progression of PD via maintenance of redox homeostasis.

1. Introduction

Parkinson's disease (PD) is a progressive condition involving dysfunction or loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). The primary symptoms of PD include difficulty with both voluntary and involuntary movements, such as bradykinesia, tremor, cogwheel rigidity and postural instability (Lew, 2007). Although the etiology of PD is still not fully understood, it is clear that neurodegeneration in this disease appears to be multi-factorial such as oxidative stress, inflammation, mitochondrial dysfunction and cellular apoptosis (Kim and Oh, 2012; Mandel et al., 2008). Although there are various methods to treat PD, present clinical treatments in PD merely exist as dopamine replacement therapy. They improve motor deficits temporarily; however, they have various adverse effects on quality of life of PD patients, such as worsened motor disorders, gastrointestinal dysfunction, dopamine dysregulation syndrome (Chaudhuri and Schapira, 2009; Houghton and Howes, 2005; Mandel et al., 2008). Thus, there is a critical unmet need for exploring new disease-

Abbreviations: Parkinson's disease, PD; substantia nigra pars compacta, SNpc; nuclear factor erythroid-2-related factor 2, Nrf2; NAD(P)H:quinone oxidoreductase 1, NQO1; heme oxygenase 1, HO-1; antioxidant response element, ARE; 6-hydroxydopamine, 6-OHDA; striatum, ST; Roswell Park Memorial Institute medium, RPMI; penicillin-streptomycin, P/S; 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT; proliferating cell nuclear antigen, PCNA; hydrogen peroxide, 3,3-diaminobenzidine, DAB; standard error of the mean, S.E.M.; reactive oxygen species, ROS; reactive nitrogen species, RNS

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modifying therapy to slow or stop the progression of PD.

The nuclear factor erythroid-2-related factor 2 (Nrf2) is a transcription factor that involves in gene expression of NAD(P)H:quinone oxidoreductase 1 (NQO1) and heme oxygenase 1 (HO-1) which are antioxidant response element (ARE) (Kim et al., 2010; Todorovic et al., 2016). In addition, it has been reported that Nrf2 stimulates the expression of Bcl2, which is a molecule that prevents cellular apoptosis. Recently, it has been reported that Nrf2 is the major cell endogenous antioxidant defense system against various diseases including neurodegenerative diseases (Johnson and Johnson, 2015). In relation to PD, Nrf2 localized in nucleus, however, its level was insufficient to delay the progression of disease in the postmortem PD patient brain compared with the healthy brain (Ramsey et al., 2007). In animal model, Nrf2 knockout mice are more susceptible to PD-induced neurotoxicity and Nrf2 activation induces the neuroprotection responses in nigral dopaminergic neurons (Chen et al., 2009). Therefore, Nrf2 is a potential target molecule in developing novel neuroprotective agents.

Ukgansan, known as *Yi-Gan San* in Chinese and *Yokukansan* in Japanese, has been used in East Asia to treat neurological disorders including neurodegenerative diseases (Hayashi et al., 2010; Ikarashi and Mizoguchi, 2016; Monji et al., 2009; Okahara et al., 2010). In particular, it has been reported that Ukgansan has the strong effects on treating PD (Shim et al., 2015). In clinical study, PD patients who treated Ukgansan for 4 weeks improve motor dysfunction as well as BPSD (Kawanabe et al., 2010). For the underlying mechanisms, there are some reports to investigate the effects of Ukgansan on PD. It was reported that Ukgansan has neuroprotective effects and rescues dopaminergic neurons from 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine toxicity, possibly via the phosphoinositide 3-kinases/Akt pathway (Doo et al., 2010). In addition, Ukgansan promotes the dopamine supplementation via inhibiting catechol-*O*-methyltransferase in rat striatum (Ishida et al., 2016). However, no report demonstrates whether the Ukgansan has effects on 6-hydroxydopamine (6-OHDA)-induced PD or not.

The present study investigates neuroprotective effects of Ukgansan against 6-OHDA-induced oxidative stress with Nrf2-mediated mechanisms. To investigate the effects of Ukgansan on 6-OHDA-induced cytotoxicity, MTT was performed in PC12 cells. For establishing underlying mechanisms related with 6-OHDA, we evaluated the levels of the translocation of Nrf2 and the expression of its mediated factors (NQO1, bcl2, bax, cytochrome C and caspase-3) in PC12 cells using immunoblotting. To evaluate whether Ukgansan ameliorates movement impairments, we conducted behavior tests with 6-OHDA-lesioned mice. In addition, we determined the effect of Ukgansan on dopaminergic neuronal death and Nrf2 activation using immunohistochemistry in 6-OHDA-induced PD mouse striatum (ST) and SNpc.

2. Experimental procedures

2.1. Preparation of Ukgansan

Ukgansan used for this study was previously described (Doo et al., 2010). Briefly, Ukgansan obtained from the Department of Pharmacy of Oriental Medicine in Kyung Hee Medical Center for the research use. 6 constituents of Ukgansan (the rhizome of *Atractylodes chinensis*, *Poria cocos*, the rhizome of *Cnidium officinale*, the radix of *Angelica acutiloba*, the radix of *Bupleurum falcatum*, and the radix of *Glycyrrhiza uralensis*) were extracted with 80% ethanol in boiling for 2 h. The hook of *Uncaria rhynchophylla* was added in the extract for last 30 min. The extract was filtered and evaporated in a rotary vacuum evaporator and lyophilized with a freezing dryer. The qualification of Ukgansan was provided previously (Doo et al., 2010).

2.2. Cell culture and drug treatment

The PC12 cell line, a rat adrenal gland pheochromacytoma, was obtained from the Korea Cell Line Bank (Seoul, Korea). Cells were maintained in RPMI [Hyclone Lab Inc (Logan, UT, USA)] with 5% heat-inactivated fetal bovine serum, 10% horse serum [Gibco Industries Inc. (Auckland, NZ)] and penicillin-streptomycin [Hyclone Lab Inc. (Logan, UT, USA)] in a water-saturated atmosphere of 5% CO2 at 37 °C. The culture medium was changed every day and PC12 cells were sub-cultured about every other day. For cell viability test and immunoblotting, PC12 cells were seeded at a density of 2.5×10^5 cells/ml or 1.05×10^6 cells/well on collagen pre-coated 96-well plates or 6-well plates, respectively. The cells were incubated for additional 24 h to adhere on the plates. The cells were pre-treated with 10 μg/ml of Ukgansan for 3 h and then exposed to 6-OHDA [Sigma-Aldrich Inc. (St. Louis, MO, USA)] at 75 μM for 8 h or 16 h.

2.3. Measurement of cell viability

Treated cells were incubated with 0.5 mg/ml of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT; Sigma-Aldrich Inc. (St. Louis, MO, USA)] at 37 °C in a CO_2 incubator for 3.5 h. MTT medium was carefully aspirated from the wells and the MTT-formazan dye was eluted using dimethyl sulfoxide (DMSO). The absorbance at 570 nm was measured using a spectrophotometer [Versamax microplate reader; Molecular Device (Sunnyvale, CA, USA)] and then expressed as a percentage of the value in the vehicle-treated control group. All the *in vitro* experiments were conducted with 6 replicates per each group.

2.4. Western blot analysis

The cells on every time point were collected and washed with ice-cold phosphate buffered saline (PBS) and then centrifuge at 2000 rpm for 10 min to obtain pelleted cells. Nuclear/cytosolic fractionation of the cells was performed by using kit [BioVision, Inc (Milpitas, CA, USA)] according to the manufacture's protocol. All the protein samples were stored at −80 °C before use. The fractions were immunoblotted as previously described (Sim et al., 2017). The separated proteins on gels were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane [Millipore (Burlington, MA, USA)]. The membranes were probed with target antibodies against Nrf2, NQO1, Bax [Abcam (Cambridge, MA, USA)]; Bcl2, cytochrome C, β-actin [Santa Cruz Biotechnology Inc. (Dallas, TX, USA)]; cleaved capspase-3 [Cell Signaling Technology, Inc. (Danvers, MA, USA)]; and PCNA [BD (Franklin Lakes, NJ, USA)]. Using respective horseradish peroxidase-conjugated secondary antibodies [Enzo Life Sciences Inc. (Farmingdale, NY, USA)] for 1 h, immunoreactive bands were developed using an enzyme-linked chemiluminescence detection kit and visualized using the ChemiDoc™ XRS + system [Bio-Rad Labs (Hercules, CA, USA)].

2.5. Animals and surgery procedure

Male ICR mice [7 weeks, 27–30 g; Daehan Biolink Co., Ltd. (Eumseong, Republic of Korea)] were housed at an ambient temperature of 23 ± 1 °C and relative humidity of 60 ± 10% under a 12 h light/dark cycle and were allowed free access to water and food. All of the experiments performed with mice were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996 and protocols approved by the Institutional Animal Care and Use Committee of Kyung Hee University (KHUASP(SE)-15-055).

Mice were anesthetized with tribromoethanol (312.5 mg/kg, i.p.) (Salazar et al., 2010) and mounted in a stereotaxic apparatus [myNeuroLab (St. Louis, MO, USA)]. Each mouse received a unilateral injection of vehicle (saline with 0.1% ascorbic acid, for sham group) or 6-OHDA (16 μg/2 μL in vehicle supplemented with 0.1% ascorbic acid), according to the following coordinates: anteroposterior (AP): 0.5 mm from bregma; mediolateral (ML): 2.0 mm from bregma; and

dorsoventral (DV): −3.0 mm from the skull (Franklin and Paxinos, 2013). The control of the flow of the injections was made by using electronic pump at 0.5 μL/min for 4 min and followed by 4 min with the needle in the injection site to avoid reflux. After surgery, mice were allowed to recover from anesthesia in a temperature-controlled chamber and then placed in individual cages.

2.6. Experimental design

Animals were divided into 4 groups (N = 12 per group) randomly as follows: (1) Sham group (Sham-operated plus intraorally vehicle-treated group); (2) Ukgansan group (Sham-operated plus intraorally Ukgansan-treated group); (3) 6-OHDA group (6-OHDA-lesioned plus intraorally vehicle-treated group); and (4) 6-OHDA + Ukgansan (6-OHDA-lesioned plus intraorally Ukgansan-treated group). 200 mg/kg of Ukgansan dissolved in normal saline were administered once per day for 10 days before the stereotaxic injection. Vehicles of equal volume were given to sham and 6-OHDA groups.

2.7. Behavioral tests

2.7.1. Pole test

The pole test is a useful method to measure bradykinesia in a mouse PD model. In the pole test, the mice were held on the top of the pole (diameter 8 mm, height 55 cm, with a rough surface) at 14 days after surgery. The time needed for the mice to climb down and place four feet on the floor was recorded as the time for locomotion activity (T-LA). Each trial had a cut off limit of 60 s.

2.7.2. Open-field test

The open-field test was conducted at 14 days after surgery. The apparatus consists of a rectangular box (40 × 25 × 18 cm) with black floor. The animals were placed in the apparatus to adapt the atmosphere for 10 min. During the test session for 30 min, track length (the distance of the mouse roaming in the center of apparatus) was estimated by a computerized automatic analysis system [Viewer; Biobserve (Bonn, Germany)]. All experiments were tested between 9 p.m. and 2 a.m. to avoid diurnal variation.

2.7.3. Apomorphine-induced rotation test

The rotation of all mice induced by apomorphine [Sigma-Aldrich Inc. (St. Louis, MO, USA)] was tested at 15 days after surgery. The mice were placed in hemispheric rotational bowl with a diameter of 40 cm. They were allowed to habituate to their environment for 5 min before the administration of apomorphine (4 mg/kg, s.c.). Full 360° turns in the direction opposite to the lesion (contralateral rotation) were counted and rotational behaviors were assessed for 25 min. All the behavioral tests were conducted by an investigator who did not know the treatment condition.

2.8. Immunohistochemistry and immunofluorescence

On the 15 days after surgery, mice were anesthetized with tribromoethanol (312.5 mg/kg, i.p.), perfused transcardially with 0.05 M PBS and then fixed with cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. Brains were removed and post-fixed in 4% PFA overnight at 4 °C and then immersed in a solution containing 30% sucrose for cryoprotection. Serial 30 μm-thick coronal sections were cut on a freezing microtome [Leica Instruments GmbH (Nussloch, Germany)] and stored in cryoprotectant (25% ethylene glycol, 25% glycerol and 0.05 M phosphate buffer) at −20 °C before use.

The tissues were stained with 3,3-diaminobenzidine [DAB; Sigma-Aldrich Inc. (St. Louis, MO, USA)] as previously described (Sim et al., 2017). Briefly, free floating brain sections were incubated overnight with a rabbit anti-TH [dilution at 1:1000; Millipore (Burlington, MA, USA)] or anti-DAT [dilution at 1:500; Millipore (Burlington, MA, USA)] antibodies after treating 1% hydrogen peroxide. Then, they were incubated with a biotinylated anti-rabbit IgG [dilution at 1:200; Vector Labs (Burlingame, CA, USA)] followed by incubation in avidin-biotin complex [ABC; Vector Labs (Burlingame, CA, USA)] solution. The color of every section was developed with DAB. The images were photographed using an optical light microscope [Olympus Microscope System BX51; Olympus (Tokyo, Japan)]. Investigators counted the number of TH-immunopositive neurons in the SNpc at × 400 magnification and measuring the optical density of TH-positive and DAT-positive fibers in the ST at × 40 magnification using Image J software [National Institutes of Health (Bethesda, MD, USA)]. Cell counting was conducted by an experimenter who did not know the treatment condition, and the result for each animal was the average of the number from its three sections.

For immunofluorescence, brain tissues were rinsed in PBS and incubated overnight with a goat anti-TH (1:1000) and rabbit anti-Nrf2 antibodies (1:300). They were then incubated with a biotinylated anti-goat IgG [dilution at 1:500; Vector Labs (Burlingame, CA, USA)] and with streptavidin-Alexa 488 [dilution at 1:500; Vector Labs (Burlingame, CA, USA)]. After rinsing PBS, sections were incubated with a goat anti-rabbit Alexa 594 [dilution at 1:500; Vector Labs (Burlingame, CA, USA)] followed by incubation in anti-Dapi [dilution at 1:5000; Vector Labs (Burlingame, CA, USA)] at room temperature. Quantification of the brain tissue sections was performed by counting the total number of dopaminergic neurons with Nrf2 translocation in the SNpc using Image J software. The Nrf2 images were visualized using confocal microscopy at × 1200 magnification [K1-Fluo; Nanoscope Systems (Daejeon, Korea)].

2.9. Statistical analysis

All statistical parameters were calculated using GraphPad Prism 5.01 software [GraphPad Software Inc. (La Jolla, CA, USA)]. Values were expressed as the mean ± standard error of the mean (S.E.M.). The results were analyzed by one-way analysis of variance followed by the Tukey's post hoc test among all groups. Differences with a p-value < 0.05 were considered statistically significant.

3. Results

3.1. Effect of Ukgansan on cell viability against 6-OHDA-induced cytotoxicity in PC12 cells

MTT assay was used to indirectly determine the potent cytotoxicity of Ukgansan on PC12 cells. As shown in Fig. 1A, Ukgansan did not show any influence on cell proliferation and cytotoxicity in PC12 cells at the different concentrations until reaching a dose of 10 μg/ml of Ukgansan treatment.

We also examined whether Ukgansan had any effect on cell viability against 6-OHDA induced cytotoxicity in PC12 cells at different concentrations (0.1–10 μg/ml) (Fig. 1B). 6-OHDA treated cells showed significantly lower cell viability (61 ± 2% relative to control group) compared to the non-6-OHDA treated group. On the other hand, Ukgansan treated groups showed significantly higher cell viability compared to the 6-OHDA only treated group regardless of Ukgansan concentrations. Because the 10 μg/ml of Ukgansan treated group (94 ± 5% relative to control group) showed the highest cell viability among all the doses, our group determined '10 μg/ml of Ukgansan' as an optimal concentration for further cellular investigation in the current study.

3.2. Effect of Ukgansan on Nrf2 translocation and protein levels of NQO1 against 6-OHDA-induced cytotoxicity in PC12 cells

To investigate the effect of Ukgansan on Nrf2 translocation, the protein levels of Nrf2 were measured both in cytoplasmic and nuclear

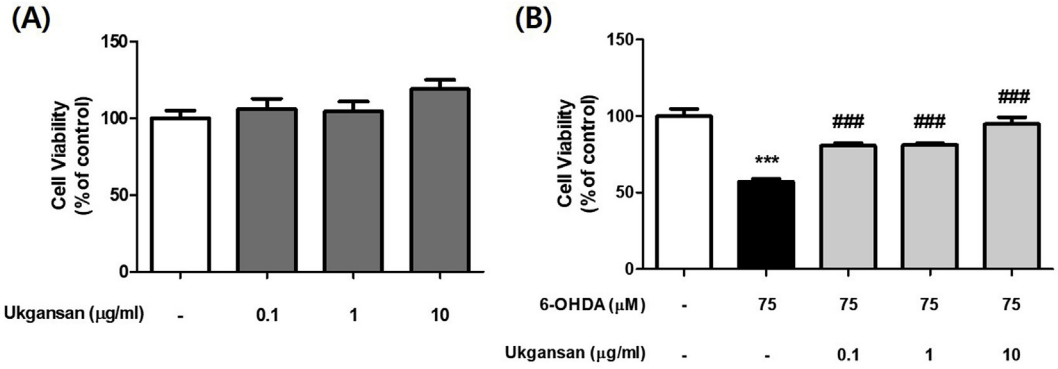

Fig. 1. Effect of Ukgansan on 6-OHDA-induced cytotoxicity in PC12 cells. (A) PC12 cells were incubated with Ukgansan at the various concentrations (0–10 μg/ml), and the cell viability was detected 24 h after incubation. (B) After treated with Ukgansan at the same concentrations in (A) for 3 h, cells were incubated with 6-OHDA (75 μM) for another 16 h. After the incubation, cell viability was examined with the MTT assay. Values are given as the mean ± S.E.M. *** $p < 0.001$; mean values were significantly different from the control group. ### $p < 0.001$; mean values were significantly different from the 6-OHDA only treated group.

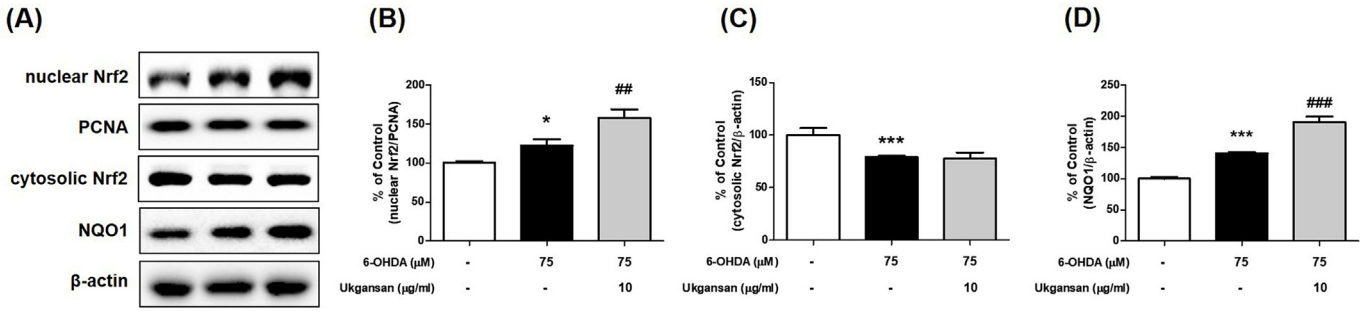

Fig. 2. Effect of Ukgansan on Nrf2 related markers in PC12 cells with 6-OHDA-induced cytotoxicity. Representative band image of Nrf2 and NQO1 from western blot analysis (A), quantitative data of the protein levels of Nrf2 in (B) nuclear fractions and (C) cytoplasmic extracts, and (D) quantitative data of the protein levels of NQO1 in the PC12 cells with 6-OHDA (8h) induced cytotoxicity in cytoplasmic extracts. Values of quantification data are given as the mean ± S.E.M. * $p < 0.05$ and *** $p < 0.001$; mean values were significantly different from the control group. ## $p < 0.01$ and ### $p < 0.001$; mean values were significantly different from the 6-OHDA only treated group.

extracts using western blotting analysis (Fig. 2). Compared to the control group (non-6-OHDA treated group), the 6-OHDA treated group showed significantly higher protein levels of nuclear Nrf2 (122 ± 13% compared to control group) but lower protein levels of cytosolic Nrf2 (79 ± 2% compared to control group). The Ukgansan pre-treated group, however, showed significantly higher protein levels of nuclear Nrf2 (158 ± 18% compared to control group) compared to the 6-OHDA group, while there were no significant differences between the 6-OHDA group and the Ukgansan group in the protein levels of cytosolic Nrf2 (78 ± 9% compared to control group).

Moreover, protein levels of NQO1 in cytosolic extract were examined to evaluate the effect of Ukgansan treatment on cell protection against 6-OHDA treatment (Fig. 2D). The protein levels of NQO1 in the 6-OHDA group (140 ± 4% compared to control group) were significantly higher than those in the control group. However, the protein levels of NQO1 in the Ukgansan group (190 ± 15% compared to control group) were significantly higher than those in the 6-OHDA group.

3.3. Effect of Ukgansan on apoptosis related markers against 6-OHDA-induced cytotoxicity in PC12 cells

To examine the effect of Ukgansan on 6-OHDA induced apoptosis, protein levels of Bcl2, Bax, cytochrome C and cleaved caspase-3 were measured (Fig. 3). The protein levels of Bcl2 in the Ukgansan group were significantly higher compared to those in the 6-OHDA group but the protein levels of Bax in the Ukgansan group were significantly lower than those in the 6-OHDA group (Fig. 3A). Subsequently, the Bcl2/Bax ratio was significantly higher in the Ukgansan group (102 ± 21% compared to control group) compared to the 6-OHDA group (64 ± 4% compared to control group) (Fig. 3B).

The 6-OHDA group showed significantly higher protein levels of cytochrome C and cleaved caspase-3 (219 ± 39% and 302 ± 78% compared to control group, respectively) compared to the control group. However, the Ukgansan group showed significantly lower protein levels of both markers (151 ± 3% and 177 ± 29% compared to control group, respectively) compared to the 6-OHDA group (Fig. 3C and D).

3.4. Effect of Ukgansan on behavioral impairment in 6-OHDA-induced PD mice models

To determine the effects of Ukgansan on 6-OHDA-induced bradykinesia, a pole test was performed (Fig. 4A). The 6-OHDA injection group significantly increased T-LA (16.8 ± 3.5 s) compared with the sham group (7.7 ± 0.4 s). However, T-LA was significantly shortened in the Ukgansan groups (9.1 ± 0.8 s).

We also performed open-field test to determine the effects of Ukgansan on 6-OHDA-induced motor impairment (Fig. 4B). The 6-OHDA injected mice significantly impaired the motor function (155 ± 8 cm) compared with the sham mice (214 ± 14 cm). However, mice treated with Ukgansan restored the motor function (209 ± 9 cm) compared with the 6-OHDA injected mice.

Unilateral 6-OHDA-injections caused an asymmetric and quantifiable motor behaviors induced by systemic administration of apomorphine. The expression of contralateral turnings after systemic injections of apomorphine is generally considered to be a typical feature of unilateral 6-OHDA-lesions (Simola et al., 2007). This contralateral

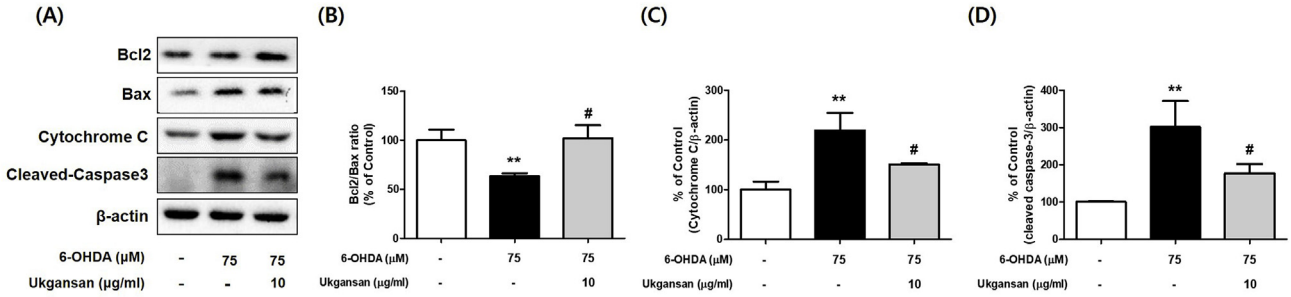

Fig. 3. Effect of Ukgansan on apoptosis against 6-OHDA-induced cytotoxicity in PC12 cells. (A) Representative band image of apoptosis related markers from western blot analysis, (B) Bcl2: Bax ratio, quantification data of (C) cytochrome C and (D) cleaved caspase-3 in PC12 cells with 6-OHDA induced cytotoxicity for 16 h. Values of quantification data are given as the mean ± S.E.M. ** $p < 0.01$; mean values were significantly different from the control group. # $p < 0.05$; mean values were significantly different from the 6-OHDA only treated group.

response is attributed to the stimulation of supersensitive dopamine receptor activation, especially in the lesioned hemisphere (Schober, 2004). In the apomorphine-induced rotation test, mice exhibited rotational behavior in a contralateral direction following apomorphine challenge 2 weeks after unilateral administration of 6-OHDA. Significant increase in the number of apomorphine-induced rotations was seen in 6-OHDA-lesioned mice (372 ± 52 turns) as compared to sham-operated mice. The Ukgansan group showed significantly decreased number of rotations (183 ± 15 turns) as compared to the 6-OHDA group (Fig. 4C).

3.5. Effect of Ukgansan against 6-OHDA-induced DA neuronal loss in ST and SNpc

To evaluate the effects of Ukgansan on dopaminergic fiber damage in ST induced by 6-OHDA, TH-immunohistochemistry in the ST of each mouse brain was performed (Fig. 5A). The 6-OHDA group had significantly fewer TH-positive fibers compared with sham group (47 ± 2% relative to the contralateral side). The Ukgansan group showed significantly lower density compared to the 6-OHDA group (59 ± 4% relative to the contralateral side).

To determine the effects of Ukgansan on dopaminergic neuronal damage induced by 6-OHDA in SNpc, TH-immunohistochemistry in the SNpc of mouse brains was performed (Fig. 5B). The 6-OHDA group had significantly fewer TH-immunopositive neurons (55 ± 3% relative to the contralateral side), whereas this reduction was significantly prevented in the Ukgansan group (68 ± 3% relative to the contralateral side) as compared to the 6-OHDA.

All of the data from immunohistochemistry were shown as representative photomicrography in Fig. 5C.

3.6. Effect of Ukgansan against 6-OHDA-induced disruption of dopamine transmission in ST

To investigate the effects of Ukgansan on the disruption of dopamine transmission induced by 6-OHDA in ST, DAT-immunohistochemistry in the ST of mouse brains were performed (Fig. 6). The 6-OHDA group showed significantly decreased DAT-positive fibers in the ST (43 ± 4% relative to the contralateral side). Treatment with Ukgansan showed restored DAT-positive fibers (57 ± 3% relative to the contralateral side) as compared to the 6-OHDA.

3.7. Effect of Ukgansan on Nrf2 translocation into nucleus of dopaminergic neurons in 6-OHDA-injected mouse SNpc

To investigate the effects of Ukgansan on Nrf2 translocation in SNpc, TH and Nrf2 double-immunofluorescences in the SNpc of mouse brains were performed (Fig. 7). The translocated Nrf2 of 6-OHDA group were significantly decreased in the ipsilateral part of SNpc (60 ± 9% compared to the sham group) compared to that of the sham-operated group. Treatment of Ukgansan with 6-OHDA-lesioned group increased Nrf2 translocation (114 ± 3% compared to the sham group) as compared to the 6-OHDA group. The activated Nrf2 of Ukgansan with sham-operated group (109 ± 16% compared to the sham group) showed no significant differences compared with that of the sham-operated group.

4. Discussion

In the present study, we investigated whether Ukgansan provides neuroprotective effects via activating Nrf2 translocation against 6-OHDA neurotoxicity in *in vitro* and *in vivo* PD model. This study clearly demonstrated that Ukgansan increased the expression of NQO1 and Bcl2 and decreased the expression of Bax, cytochrome C and cleaved

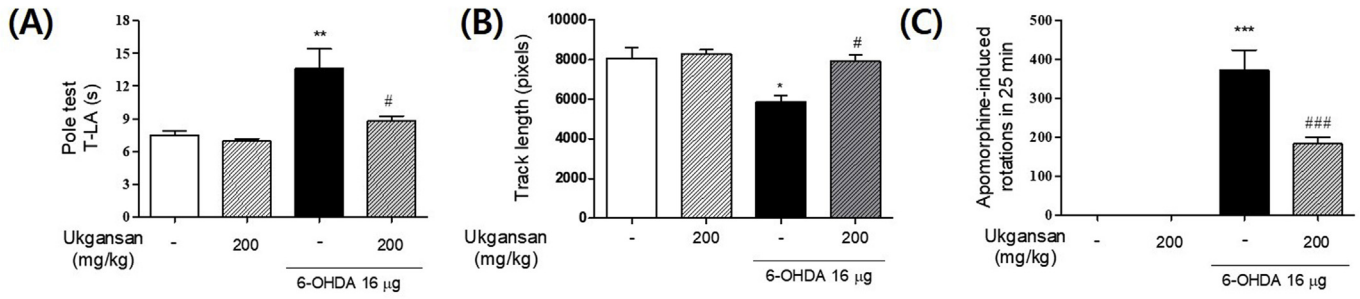

Fig. 4. Effect of Ukgansan on movement impairment in 6-OHDA-induced PD mice model. (A) The time to turn and arrive at the floor (T-LA) was recorded, with the cut-off limit of 60 s in the pole test. (B) In the open-field test, track length was recorded. (C) Contralateral turns per 25 min that measured for 25 min after apomorphine injection (4 mg/kg, s.c.). Values are indicated as the mean ± SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$; mean values were significantly different from the sham group. # $p < 0.05$ and ### $p < 0.001$; mean values were significantly different from the 6-OHDA group.

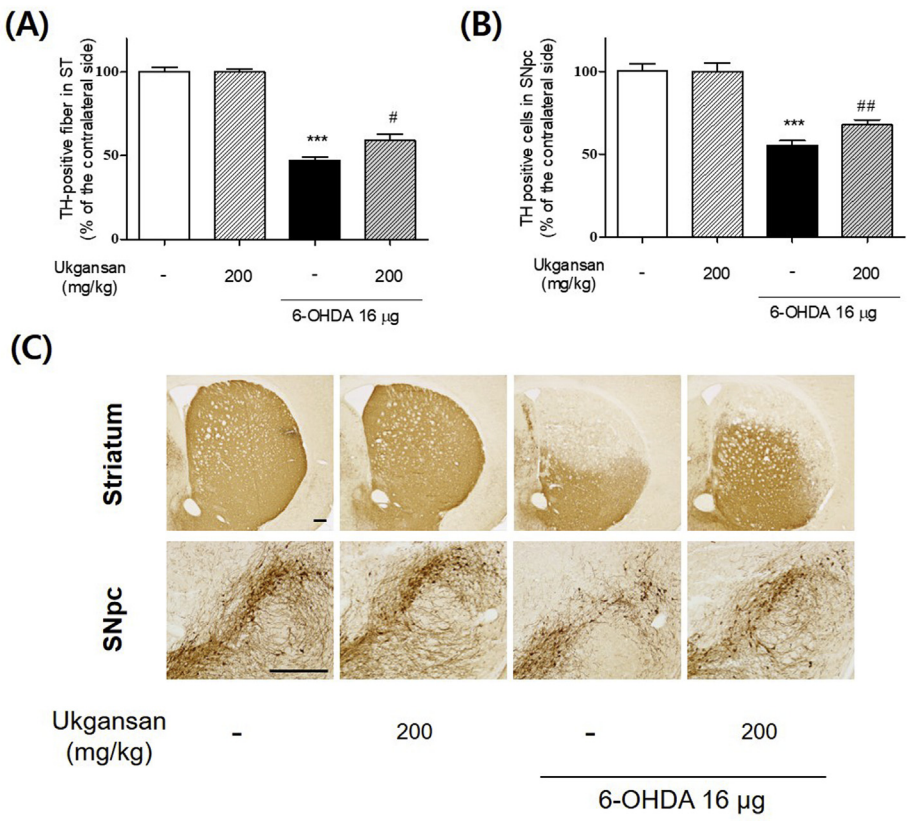

Fig. 5. Effect of Ukgansan on dopaminergic neuronal loss in the ST and SNpc of mice. (A) The optical density of TH-positive fibers in the ST and (B) the number of TH-positive neurons in the SNpc in 6-OHDA induced PD mice model. (C) Representative photomicrographs of (A) and (B). *** $p < 0.001$; mean values were significantly different from the sham group. # $p < 0.05$ and ## $p < 0.01$; mean values were significantly different from the 6-OHDA group. Scale bar = 200 μm.

caspase-3 by activating Nrf2 translocation, resulting in the neuroprotection against 6-OHDA in PC12 cells. In addition, our study showed that Ukgansan ameliorated behavioral impairments and prevented dopaminergic neuronal loss in 6-OHDA-lesioned mice via activating Nrf2 in SNpc. These findings suggest that the motor improvement after treatment with Ukgansan might be mediated by its Nrf2 activating effects.

As mentioned previously, PD has been recognized as a progressive condition that involves dopaminergic neuronal dysfunction or loss in the SNpc and ST (Izumi et al., 2015; Zhang et al., 2013). Oxidative stress is widely suspected as a key factor involving damage of dopaminergic neurons (Barnham et al., 2004). It is generally recognized that oxidative stress from unregulated reactive oxygen species (ROS) and reactive nitrogen species (RNS) can damage the cells including neuronal cells, the deterioration of mitochondria and activation of apoptotic caspase cascades (Tobon-Velasco et al., 2013). Although the brain consumes approximately 20% of the total oxygen supply of the body (Johnson et al., 2012) and is composed of a large amount of

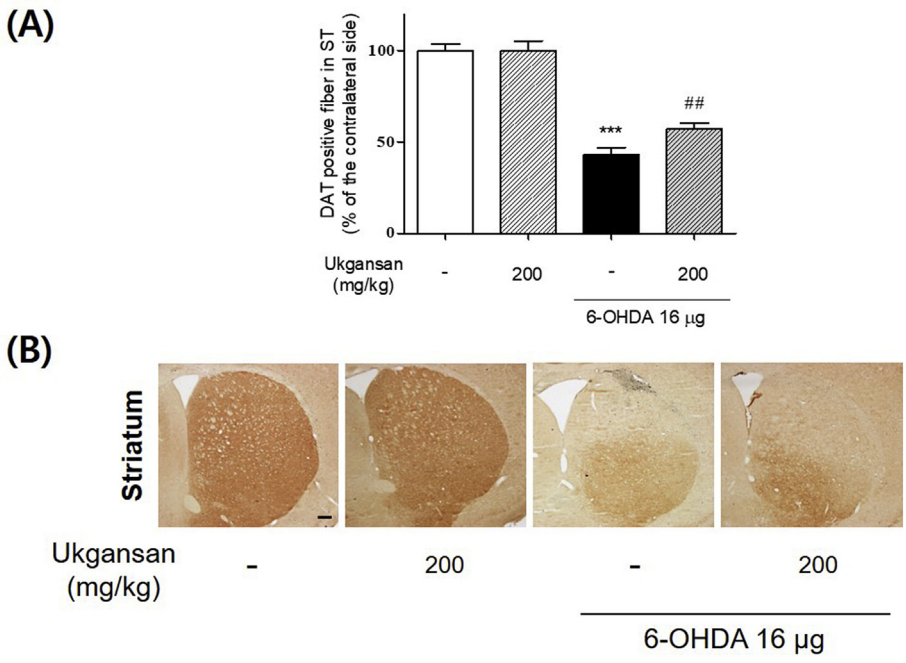

Fig. 6. Effect of Ukgansan on dopamine transmission in the ST of mice. (A) The optical density of DAT-positive fibers in the ST of 6-OHDA induced PD mice model. (B) Representative photomicrographs. *** $p < 0.001$; mean values were significantly different from the sham group. # $p < 0.05$ and ## $p < 0.01$; mean values were significantly different from the 6-OHDA group. Scale bar = 200 μm.

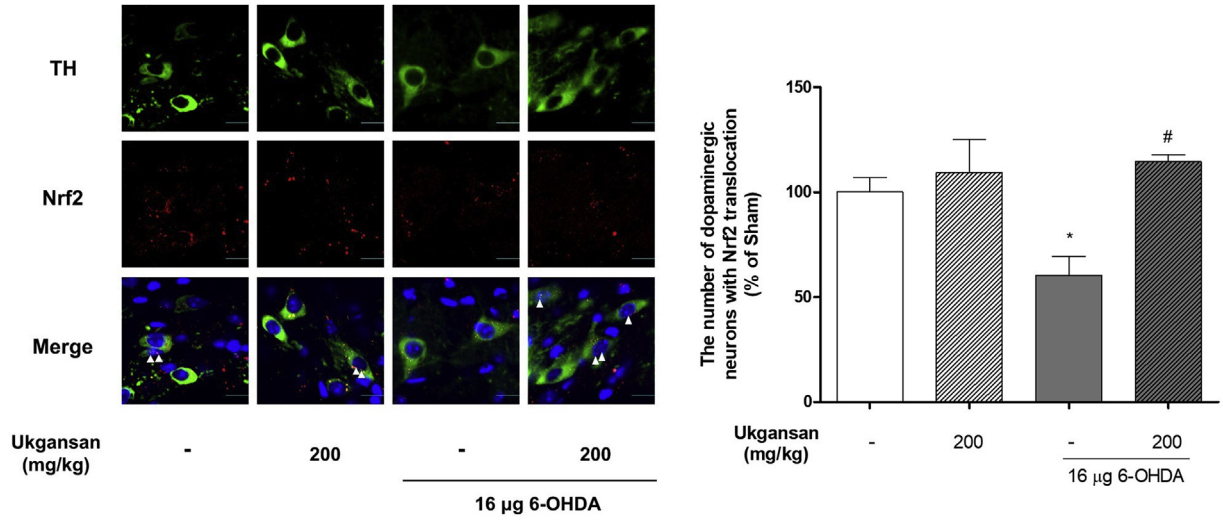

Fig. 7. Effect of Ukgansan on Nrf2 translocation into nucleus of dopaminergic neurons in 6-OHDA-injected mouse SNpc. The translocated Nrf2 into nucleus of dopaminergic neurons in the mouse SNpc (white arrow). Representative photomicrographs (Right). * $p < 0.05$; mean values were significantly different from the sham group. # $p < 0.05$; mean values were significantly different from the 6-OHDA group. Scale bar = 20 μm.

polyunsaturated fatty acids (Galli et al., 1977), SN is considerably more vulnerable to oxidative stress for its inherently less antioxidant glutathione compared to several parts of the brain (Loeffler et al., 1994). In PD, dopamine auto-oxidation and mitochondrial dysfunction are primary reasons for producing ROS in dopaminergic cells (Jiang et al., 2014). For these reasons, it might be important to ameliorate antioxidant defense system to control the oxidative stress at the cellular level.

Nrf2 is a major transcription factor that involves in gene expression of antioxidant defense system factors, including NQO1 (Todorovic et al., 2016). In this context, we examined Nrf2 translocation in PC12 cells with 6-OHDA induced cytotoxicity. As shown in Fig. 2A and B, 6-OHDA induced Nrf2 translocation in PC12 cells, however, Ukgansan was considerably more likely stimulate Nrf2 translocation compared to the 6-OHDA only treated group. In addition, the protein levels of NQO1 in all of the groups showed a similar pattern regarding Nrf2 translocation. This phenomenon could be understood as a compensatory effect of Nrf2 and there have been several reports that xenobiotics or toxic insults increased Nrf2 translocation as compensatory cellular reactions to protect themselves (Izumi et al., 2015; Zhang et al., 2013). Furthermore, it has been elucidated that Nrf2 up-regulates the expression of anti-apoptotic protein 'Bcl2' and consequently prevents cellular apoptosis (Niture and Jaiswal, 2012). However, as shown in Fig. 3, the ratio between Bcl2 and Bax in 6-OHDA groups showed the reversed pattern compared to Nrf2 translocation in 6-OHDA groups, since the activation of Nrf2 by 6-OHDA toxicity was insufficient to halt the degradation of cells against oxidative damage. At the same time, the current study examined the protein levels of further downstream cascades, which include apoptotic molecules, such as cytochrome C and cleaved caspase-3 (Rosse et al., 1998). The 6-OHDA treatment increased pro-apoptotic cascades including cytochrome C and caspase-3 in PC12 cells. However, the Ukgansan pretreatment reduced the protein levels of cytochrome C and cleaved caspase-3 in dopaminergic cells with 6-OHDA induced cytotoxicity. With our present data, it suggests that Ukgansan effectively utilized Nrf2 translocation to accelerate the expression of cell protective molecules such as NQO1 and Bcl2 and consequently suppressed 6-OHDA induced apoptosis in dopaminergic cells.

The current study further investigated the effects of Ukgansan treatment on PD symptoms and dopaminergic neurons in 6-OHDA-lesion induced PD mice model through the evaluation of movement impairments and dopaminergic neuronal loss in ST and SNpc, respectively. The 6-OHDA-lesion induction model has been widely used in PD research with rodents, as this model is reliable and shows robust motor disorders (da Conceicao et al., 2010). We examined movement impairments, which were generally observed in mice with PD. The pole test has been used for the last several decades to evaluate mouse movement disorders caused by striatal dopamine depletion. These disorders include bradykinesia *in vivo* animal PD models (Matsuura et al., 1997). In the pole test, Ukgansan pre-administration showed improvement in the T-LA and track length compared to the 6-OHDA-only treatment. In addition, Ukgansan ameliorated side-based motor impairments induced by apomorphine injections. Moreover, our group measured apomorphine-induced rotation because the contralateral turning after apomorphine injection is one of the typical and important features of unilateral 6-OHDA-lesions (Schober, 2004; Simola et al., 2007). As shown in our results, Ukgansan pretreatment significantly reduced apomorphine-induced rotations in 6-OHDA-lesioned mice in the current study.

As these motor symptoms in PD are ascribed to striatal damage derived from cell loss of SNpc (Dauer and Przedborski, 2003), the current study examined dopaminergic cell loss in ST and SNpc, as well as dysfunction in ST, through the expression of TH and DAT, respectively. In the current study, it was observed that 6-OHDA-lesion reduced TH expression in both ST and SNpc as well as decreasing DAT expression in ST region. However, our results elucidated that Ukgansan treatment protected dopaminergic cell loss in both ST and SNpc, which was demonstrated by TH expression in both ST and SNpc. In addition, Ukgansan treatment protected cell functions of ST from 6-OHDA induced damage that was shown through DAT expression. Moreover, we confirmed the activation of Nrf2 in dopaminergic tissues in 6-OHDA lesion induced PD models. These histological data compare well alongside behavioral data. With the data from *in vitro* study, it might be suggested that Ukgansan pre-administration protects dopaminergic cells from 6-OHDA.

Taken together, the results of the current study demonstrated protective effects of Ukgansan on dopaminergic neurons via stimulating of Nrf2 translocation and its related cell protective mechanisms, including the NQO1 and Bcl2 family, against 6-OHDA toxicity *in vitro* and *in vivo*. Consequently, through the suppression of dopaminergic neuronal loss in ST and SNpc, Ukgansan ameliorated motor deficits in 6-OHDA-injected mice. These results provide potent evidence that Ukgansan might prevent PD progression as the neuroprotective agents.

Contribution of authors

H. Eo and M. S. Oh designed and coordinated this study. H. Eo, E. Huh and Y. Sim performed the experiments and analyzed the data. H. Eo, E. Huh and M. S. Oh wrote the manuscript. All the authors participated in discussion of the results and reviewed the final draft.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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