RESEARCH ARTICLE

Korean red ginseng promotes hippocampal neurogenesis in mice

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Abstract

Neurogenesis in the adult hippocampus plays a major role in cognitive ability of animals including learning and memory. Korean red ginseng (KRG) has long been known as a medicinal herb with the potential to improve learning and memory; however, the mechanisms are still elusive. Therefore, we evaluated whether KRG can promote cognitive function and enhance neurogenesis in the hippocampus. Eight-week-old male C57BL/6 mice received 50 mg/kg of 5-bromo-2′-deoxyuridine (BrdU) intraperitoneally and 100 mg/kg of KRG or vehicle orally once a day for 14 days. Pole, Rotarod and Morris water maze tests were performed and the brains were collected after the last behavioral test. Changes in the numbers of BrdU- and BrdU/doublecortin (DCX; a marker for neuronal precursor cells and immature neurons)-positive cells in the dentate gyrus and the gene expression of proliferating cell nuclear antigen (a marker for cell differentiation), cerebral dopamine neurotrophic factor and ciliary neurotrophic factor in the hippocampus were then investigated. KRG-treated mice came down the pole significantly faster and stood on the rotarod longer than vehicle-treated mice. The Morris water maze test showed that KRG administration enhanced the learning and memory abilities significantly. KRG also significantly increased BrdU- and BrdU/DCX-positive cells in the dentate gyrus as well as the proliferating cell nuclear antigen, cerebral dopamine neurotrophic factor and ciliary neurotrophic factor mRNA expression levels in the hippocampus compared to vehicle. Administration of KRG promotes learning and memory abilities, possibly by enhancing hippocampal neurogenesis. This study was approved by the Pusan National University Institutional Animal Care and Use Committee (approval No. PNU-2016-1071) on January 19, 2016.

Key Words: *bromodeoxyuridine; cerebral dopamine neurotrophic factor; ciliary neurotrophic factor; doublecortin; ginseng; hippocampus; Korean red ginseng; learning; memory; neurogenesis; proliferating cell nuclear antigen; red ginseng*

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Introduction

In the dentate gyrus, new neurons are generated by undergoing developmental stages of differentiation during adulthood. In the subgranular zone of the dentate gyrus, adult neural stem cells generate intermediate progenitor cells, giving rise to neuroblasts that develop into neurons (Goncalves et al., 2016).

The functional significance of adult hippocampal neurogenesis has been demonstrated in cognitive ability including learning, memory, exercise and antidepressants (Sahay et al., 2011). Newly generated neuronal cells in the adult hippocampus are functionally combined with the existing neuronal circuitry, which are positively engaged in the hippocampus-dependent processes of learning and memory functions (Sahay et al., 2011). Recent studies have demonstrated that learning and memory are facilitated in mice with more newborn neurons (Sahay et al., 2011) and the newborn neurons influence encoding of information (Snyder et al., 2011), while suppression of neurogenesis in the dentate gyrus impairs spatial pattern separation in mice (Clelland et al., 2009) and recognition memory in rats (Jessberger et al., 2009). Therefore, it is assumed that agents enhancing neurogenesis in the hippocampus promote the learning and memory abilities.

Panax ginseng Meyer (ginseng), which is one of the major medicinal herbs grown in Korea, has been widely used for many centuries as a general restorative for human health (Oliynyk and Oh, 2013), as well as a therapeutic for treating diverse conditions including neurodegenerative disease (Jun et al., 2015), inflammatory disease and cancer (Ryu et al., 2016). Recent studies have shown that ginseng administration improves cognitive function (Geng et al., 2010) and working memory (Reay et al., 2010) in healthy participants, hematological indices were within the normal range after four weeks of ginseng oral administration (Lee et al., 2012) and serious adverse events were not found during ginseng administration (Geng et al., 2010), indicating that ginseng can improve cognitive function with safe in humans.

The composition of ginseng can vary depending on processing methods (Kang et al., 2006). Red ginseng is a kind of processed ginseng by steaming and drying, which imparts

newly formed pharmacological properties through heat-induced chemical transformation (Park, 1996; Konoshima et al., 1998). Red ginseng ameliorates learning and memory deficits in old and young rats (Zhong et al., 2000) as well as in aged mice (Lee and Oh, 2015). Korean red ginseng (KRG), the steamed and dried root of ginseng cultivated in Korea, exerts significant therapeutic effects in Parkinson's disease (Bach et al., 2016; Ryu et al., 2016; Kim et al., 2018) and ischemia (Ban et al., 2012) models. Moreover, it enhances human motor and cognitive function in healthy subjects (Yeo et al., 2012), indicating that KRG can improve not only motor but also learning and memory functions; however, it is still not clear that KRG can enhance neurogenesis and regulate the expressions of neurogenesis-related neurotrophic factors including cerebral dopamine neurotrophic factor (CDNF) and ciliary neurotrophic factor (CNTF) in the hippocampus.

Because of the correlation between neurogenesis in the hippocampus and the abilities of learning and memory, we hypothesized that KRG may enhance hippocampal neurogenesis that is related to the improvement of cognitive function. Therefore, we investigated whether KRG can improve motor and cognitive behaviors and promote neurogenesis in adult hippocampus of C57BL/6 mice in this study and explore the underlying mechanisms.

Materials and Methods

Animals

This study was approved by the Pusan National University Institutional Animal Care and Use Committee (approval No. PNU-2016-1071) on January 19, 2016. Male 8-week-old C57BL/6 mice weighing 20–22 g were purchased from Orient Bio Inc. (Seongnam, Korea) and housed at 22 ± 2°C in a light-controlled environment with free access to food and water *ad libitum*. After a 7-day adjustment period, mice were randomly assigned to two groups (n = 9 per group): a vehicle-treated group (Veh) and a 100 mg/kg KRG-treated group (KRG).

KRG administration and bromodeoxyuridine injection

The KRG extract was acquired from the Korea Ginseng Corporation (Daejeon, Korea). After a metal gavage needle was inserted into the esophagus of a mouse, vehicle or KRG extract was administered to the mouse. Mice in the KRG group orally received 100 mg/kg of KRG extract diluted with sterilized water once a day for 14 consecutive days, while those in the Veh group received 0.1 mL of vehicle on the same schedule. After all the oral administration was finished, all mice received intraperitoneal injection of 5-bromo-2′-deoxyuridine (BrdU; 50 mg/kg; Sigma, St. Louis, MO, USA) once a day for 14 consecutive days to detect cell mitosis. The entire time schedule in this study is shown in **Figure 1**.

Pole test

The pole test is a behavioral test for evaluating motor function. Mice (n = 6 per group) were placed on the top of a rough-surfaced wood pole measuring 10 mm in diameter and 55 cm in height, and the time taken to reach the floor was measured. The test was repeated three times with an interval of 30 seconds and the average of the descending times was calculated. The test was conducted on days 0, 7 and 14.

Rotarod test

The rotarod test is used to assess motor learning as well as sensorimotor coordination in rodent models (Laurer et al., 2001). Before the test, each mouse (n = 6 per group) was trained five times per day for 3 days on the rod with a constant speed of 10 r/min. During the test session, mice were placed on the cylinder, and the time for which the animal stayed on the cylinder was measured. The constant speed (r/min) was slowly increased from 4 to 40 within 5 minutes. The test was stopped if the mouse fell or the latency to fall reached 5 minutes. During the test session, each mouse was tried to three times with an interval of 30 minutes, and the average of the latency to fall was calculated. The test was performed on days 0, 4, 7 and 14.

Water maze test

The Morris water maze (MWM) was conducted in a circular pool measuring 120 cm in diameter and 45 cm in height containing nontoxic white-colored water maintaining at 23–25°C. A hidden white platform measuring 10 cm in diameter and 30 cm in height was placed in one of the quadrants with equal area and submerged 2 cm below the water surface. During the 6 subsequent days (from day 8 to 13) of training, the mice (n = 6 per group) were tried to three times per day

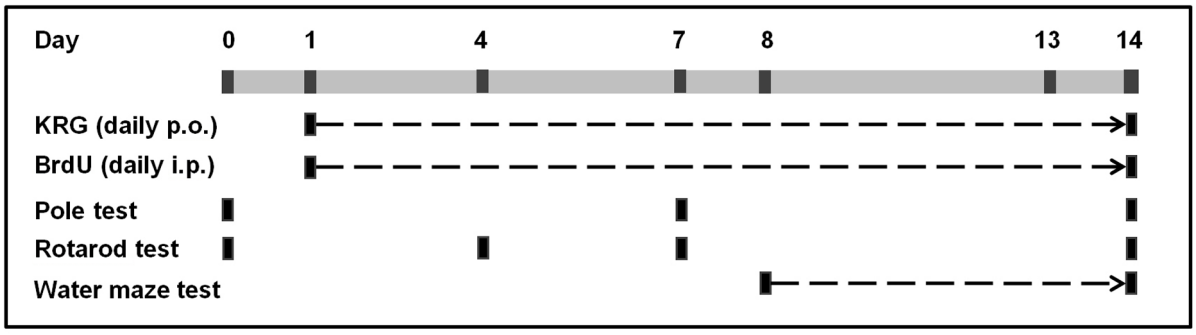

Figure 1 Schedule of experiment.
From day 1, the mice were orally (p.o.) administered vehicle or Korean red ginseng (KRG) and injected with 50 mg/kg of 5-bromo-2′-deoxyuridine (BrdU) intraperitoneally (i.p.) per day for 14 days. The pole test was performed on days 0 (1 day prior to the first administration of KRG), 7 and 14. The rotarod test was performed on days 0, 4, 7 and 14. The Morris water maze test was conducted from 8 to 14 days.

with the platform in the pool. Start position was randomly selected and the mouse was guided to the platform if it did not find the platform within 90 seconds (Yin et al., 2017). The escape latency spent to find the platform was monitored during each trial session by a video tracking system using the S-MART 2.5 software (PanLab, Barcelona, Spain). On the last day (day 14), the hidden platform was removed from the water-maze tank and a probe test was performed. Mice were allowed to swim for 90 seconds and the number of platform location crossings, the staying time and the swimming distance in the hidden platform and the maze quadrant where the platform had previously been located were recorded using a video tracking system (PanLab).

Immunohistochemistry

Mice were perfused transcardially with 4% paraformaldehyde dissolved in 0.1 M phosphate buffer after the last behavioral test and their brains were harvested. Frozen sections were cut to a thickness of 20 μm, after which sections were respectively incubated with 2 N HCl at 37°C for 10 minutes, 0.1 M boric acid at room temperature for 3 minutes and anti-BrdU (1:200; Abcam, Cambridge, UK) and anti-double-cortin (DCX; 1:100; Abcam) primary antibodies overnight at 4°C. The sections were re-incubated at room temperature for 2 hours with Alexa 488-conjugated IgG (1:200; Molecular Probes, Eugene, OR, USA) and Alexa 568-conjugated IgG (1:200; Molecular Probes) secondary antibodies.

The stained sections were captured with a confocal microscope (Carl ZEISS DE/LSM700, Öberkochen, Germany). The numbers of BrdU-positive cells and BrdU/DCX double-labeled cells in the dentate gyrus on each capture were manually counted by two independent observers to minimize the possibility of observer bias and the counted value was divided by the dentate gyrus area on each capture. The average value of the counts was calculated in three continuous hippocampal sections. Positive cell rate was calculated as the average value of positive cells in the KRG group versus the average value of positive cells in the Veh group.

Real-time quantitative RT-PCR

After mice were sacrificed, the bilateral hippocampi were immediately removed and frozen in liquid nitrogen. To determine the proliferating cell nuclear antigen (PCNA), CDNF and CNTF, the hippocampi were homogenized and real-time quantitative polymerase chain reaction (qPCR) analysis was performed. The PCR primers used in this study were synthesized commercially (Bioneer, Daejeon, Korea) as follows: PCNA (forward, 5′-TTTGAGGCACGCCTGATCC-3′; reverse, 5′-GGAGACGTGAGACGAGTCCAT-3′), CDNF (forward, 5′-GGTCGCTAAAATTGCAGAGC-3′; reverse, 5′-AAGGTAGCCCAGCCCACTAT-3′), CNTF (forward, 5′-GGGACCTCTGTAGCCGCTCTATCTG-3′; reverse, 5′-GTTCCAGAAGCGCCATTAACTCCTC-3′) and glyceraldehyde-3-phosphatedehydrogenase (GAPDH; forward, 5′-GGCATTGCTCTCAATGGACAA-3′; and reverse, 5′-CCGAGGTTGGGATAGGGCC-3′). The cDNA amplification was performed using the Maxima SYBR Green qPCR Master Mix (Applied Biosystems).

Statistical analysis

The behavioral data of the pole test, the rotarod test and the escape latency time spent to find the hidden platform were expressed as the mean ± SEM and other data were expressed as the mean ± SD. Mann-Whitney *U* test was used to compare behavioral data, cell counts and mRNA expression between groups. Prism 5 for Windows (GraphPad Software Inc., La Jolla, CA, USA) was used for all statistical analyses and $P < 0.05$ was considered statistically significant.

Results

Effect of KRG on motor function

In the pole test, there was no significant difference in the descending time between the Veh and KRG groups before KRG administration. However, the descending time in the KRG group was significantly shorter than that in the Veh group after 7 and 14 days of KRG administration ($P < 0.05$; **Figure 2A**).

A similar tendency was observed in the rotarod test. Before KRG administration, there was no significant difference in the time for which the animal remained on the rotarod between the Veh and KRG groups. However, repeated KRG administrations led to a gradual increase in this time and there were significant differences in the time for which the animal remained on the rotarod between groups on days 7 and 14 ($P < 0.05$; **Figure 2B**).

Effects of KRG on learning and memory abilities

The MWM was employed to investigate the possible effects of KRG treatment on learning and memory abilities. Until the 4th training day (from day 8 to day 11), there was no significant difference in the escape latency (time spent to find the hidden platform) between the Veh and KRG groups. However, mice in the KRG group found the platform significantly faster than those in the Veh group on the 5th and 6th training days (days 12 and 13; $P < 0.05$ at each day; **Figure 3A**).

On the 7th training day (day 14), the hidden platform was removed and a probe test was performed. Mice in the KRG group crossed the platform location significantly more often than those in the Veh group ($P < 0.05$; **Figure 3B**). Moreover, mice in the KRG group stayed on the hidden platform ($P < 0.05$; **Figure 3C**) and in the maze quadrant ($P < 0.05$, **Figure 3F**) where the platform had previously been located longer than those in the Veh group. Additionally, the swimming distance of mice in the KRG group was significantly longer than that of mice in the Veh group in these locations ($P < 0.05$; **Figure 3C–E**, **G**, and **H**).

Effects of KRG administration on cell differentiation

After 14 days of KRG treatment, the number of BrdU-positive cells in the granular cell layer was significantly increased in the KRG group compared with the Veh group ($P < 0.05$; **Figure 4A** and **B**), and the level of PCNA mRNA in the KRG

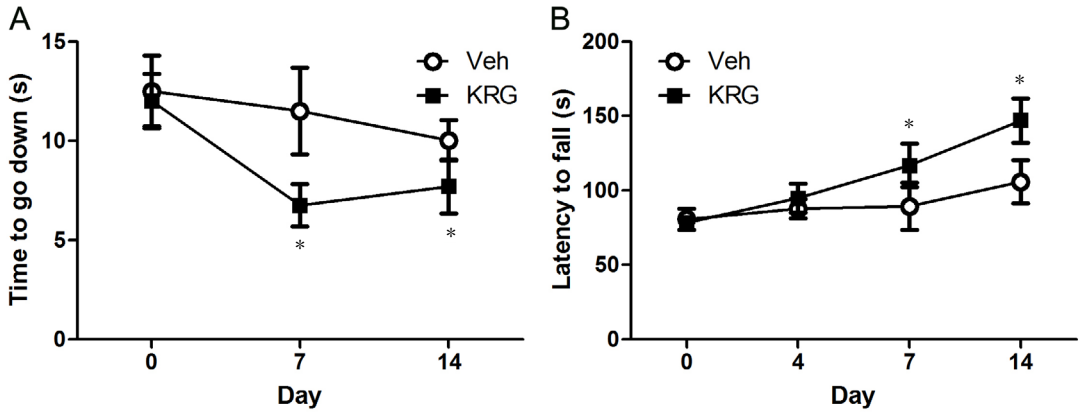

Figure 2 Behavioral changes in response to KRG based on the pole and rotarod tests.
(A) Results of the pole test. KRG-treated mice arrived at the floor significantly faster than vehicle-treated mice on days 7 and 14. (B) Results of the rotarod test. KRG-treated mice significantly stayed longer than vehicle-treated mice on days 7 and 14. Data are shown as the mean ± SEM (n = 6 per group). $*P$ < 0.05, *vs.* Veh-treated group (Mann-Whitney *U* test). KRG: Korean red ginseng; Veh: vehicle.

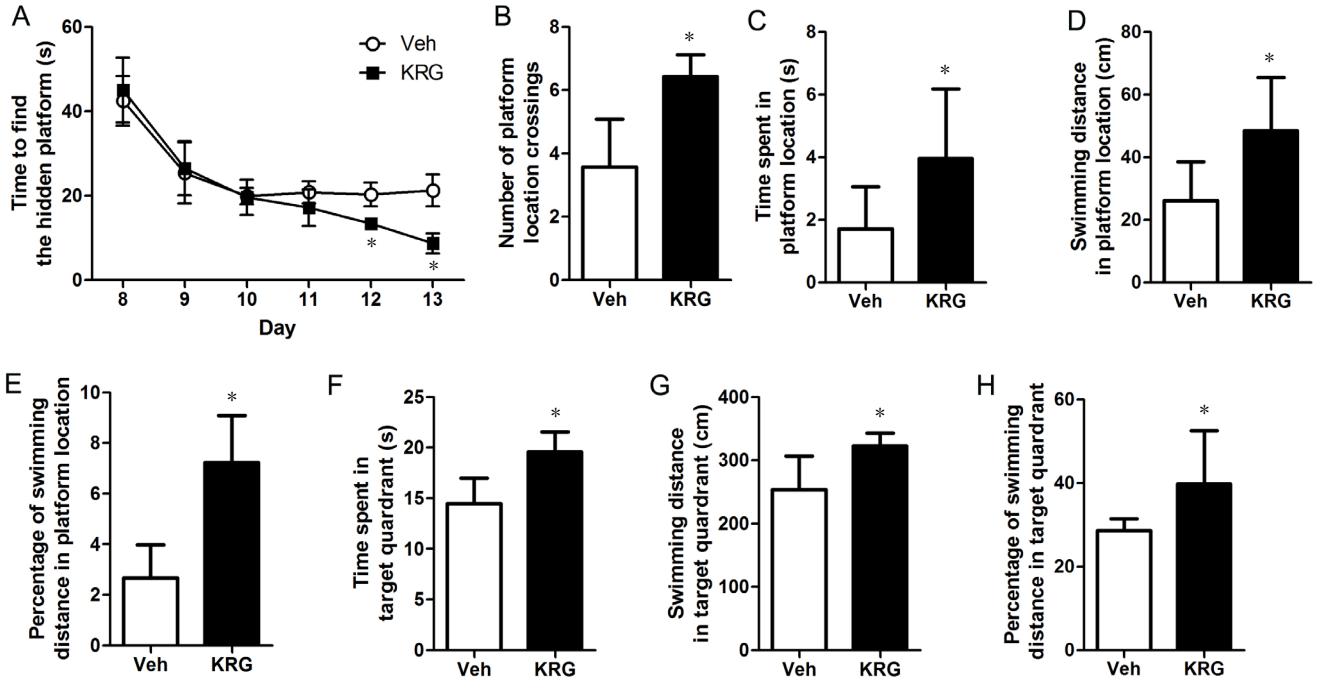

Figure 3 Results of the Morris water maze test.
(A) Mice in the KRG group found the platform significantly faster than those in the Veh group on the 5th and 6th training days (on days 12 and 13). On the 7th day (day 14), mice in the KRG group crossed the platform location significantly more often than those in the Veh group (B). Moreover, mice in the KRG group stayed on the hidden platform (C) and in the quadrant (F) in which the platform had previously been located significantly longer than those in the Veh group. The swimming distance of mice in the KRG group was significantly longer than that of mice in the Veh group in the area of the hidden platform (D, E) and the maze quadrant in which the platform had been located (G, H). The escape latency spent to find the hidden platform are expressed as the mean ± SEM, and the remaining data are shown as the mean ± SD (n = 6 per group). $*P$ < 0.05, *vs.* Veh group (Mann-Whitney *U* test). KRG: Korean red ginseng; Veh: vehicle.

group was significantly higher than that in the Veh group (P < 0.05; **Figure 5A**), suggesting that KRG can enhance cell proliferation in the hippocampus. Moreover, the number of BrdU/DCX-positive cells in the dentate gyrus of the KRG group was significantly increased compared with the Veh group (P < 0.05; **Figure 4C**), suggesting that KRG administration promoted the differentiation and expansion of neuroblasts in the granular cell layer.

Effects of KRG administration on changes in neurotrophic factors in the hippocampus

Real time qPCR analysis was performed for screening of influencing factors related to KRG administration. The results revealed that KRG administration increased the levels of CDNF (P < 0.05; **Figure 5B**) and CNTP (P < 0.05; **Figure 5C**) in the hippocampus, suggesting that these factors play crucial roles in KRG-induced neurogenesis in the hippocampus.

Discussion

The results of this study demonstrate that KRG treatment significantly promoted motor behaviors and cognitive development. Moreover, KRG treatment significantly increased not only neuroblasts by enhancing cell differentiation but also the expression of CDNF and CNTF mRNA in

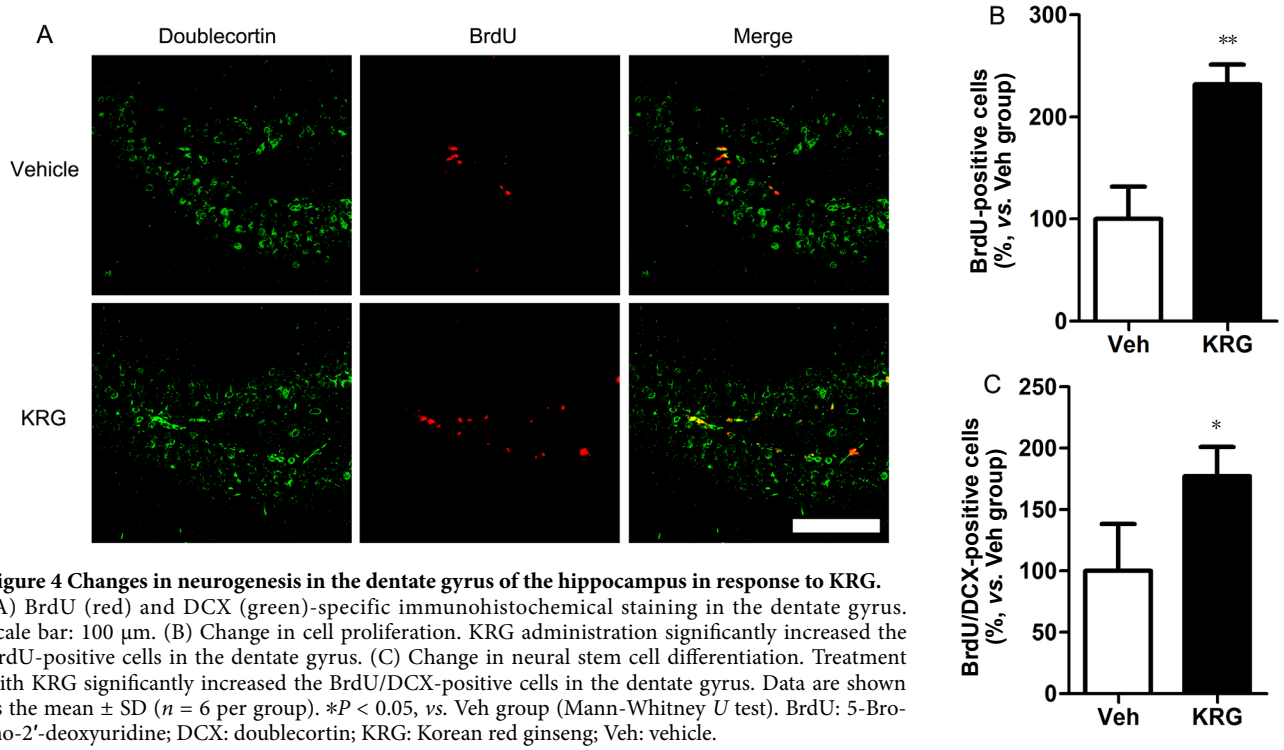

Figure 4 Changes in neurogenesis in the dentate gyrus of the hippocampus in response to KRG.
(A) BrdU (red) and DCX (green)-specific immunohistochemical staining in the dentate gyrus. Scale bar: 100 μm. (B) Change in cell proliferation. KRG administration significantly increased the BrdU-positive cells in the dentate gyrus. (C) Change in neural stem cell differentiation. Treatment with KRG significantly increased the BrdU/DCX-positive cells in the dentate gyrus. Data are shown as the mean ± SD (n = 6 per group). $*P < 0.05$, *vs.* Veh group (Mann-Whitney U test). BrdU: 5-Bromo-2′-deoxyuridine; DCX: doublecortin; KRG: Korean red ginseng; Veh: vehicle.

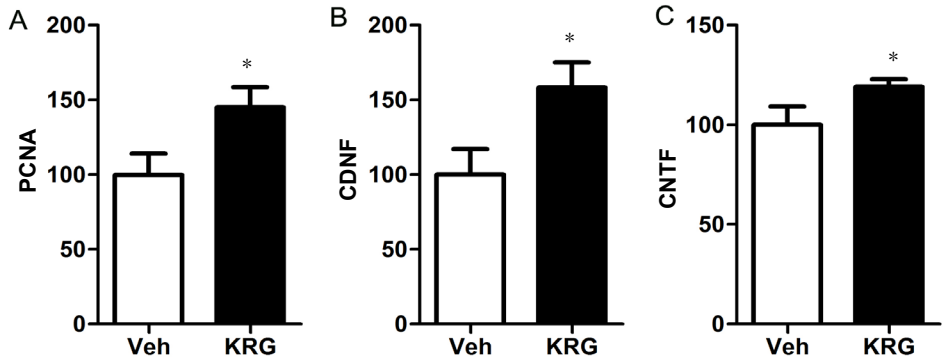

Figure 5 Real-time qPCR analysis of PCNA, CDNF and CNTF.
Administration of Korean red ginseng significantly increased the relative mRNA levels of PCNA (A), CDNF (B), and CNTF (C). Data are shown as the mean ± SD (n = 3 per group). $*P < 0.05$, *vs.* Veh group (Mann-Whitney U test). CDNF: Cerebral dopamine neurotrophic factor; CNTF: ciliary neurotrophic factor; KRG: Korean red ginseng; PCNA: proliferating cell nuclear antigen; qPCR: quantitative polymerase chain reaction; Veh: vehicle.

the hippocampus.

Traditionally ginseng had been known to improve mental functions and physical performance, and actually studies have shown that ginseng or ginsenoside complex administration improve physical performance of healthy adults (Caldwell et al., 2018; Lee et al., 2018) as well as cognitive and memory abilities (Oliynyk and Oh, 2012). To verify whether ginseng improves motor function, the pole and the rotarod tests were performed. The pole test was developed to evaluate motor dysfunction in rodent models of Parkinson's disease (Matsuura et al., 1997) and focal ischemia (Balkaya et al., 2013). The rotarod test was developed to assess the effect of drugs on rodent behaviors and can be used for rodent models of brain diseases including Huntington's disease (Dunnett and Brooks, 2018) and traumatic brain injury (Mouzon et al., 2012). Both tests are useful for the evaluation of motor function of rodents because the pole test is a useful tool to evaluate the mouse motor function including grasp and maneuver on a pole and the rotarod test is a widely used tool for evaluating the motor function including motor coordination, grip strength, and balance of rodents (Dela Pena et al., 2017). In this study, KRG-treated mice arrived faster at the floor and remained longer on the rotarod, which indicate that KRG can promote motor function.

To evaluate whether KRG enhances learning and memory functions, the MWM test was performed. The MWM test is a behavioral test generally used to investigate learning and memory abilities in which the mouse or rat tries to find a visible or invisible platform that allows the animals to avoid the water using several cues (Vorhees and Williams, 2006). In this study, KRG-treated mice found the platform faster, crossed the platform location more often and stayed longer in the hidden platform and the maze quadrant where the platform had been located previously than vehicle-treated mice, indicating that KRG enhances learning and memory abilities. Previous studies have reported that KRG contains

substances capable of improving learning and memory abilities in aged mouse models (Lee and Oh, 2015), enhances the short term spatial working memory in an animal model of autism (Gonzales et al., 2016), and significantly ameliorates scopolamine-induced memory impairment (Dela Pena et al., 2017), which supports the results of the present study showing that KRG improves learning and memory abilities.

The hippocampus plays a crucial role in learning and memory abilities (Snyder et al., 2005) and the newly generated neurons in the granular cell layer of the dentate gyrus play a particularly important role in these abilities (Deng et al., 2010). Therefore, we investigated whether KRG administration could enhance neurogenesis in the dentate gyrus and if the cells differentiated in response to KRG were neuroblasts. Oral administration of KRG increased the number of BrdU-positive cells and the expression of PCNA mRNA in the hippocampus. Moreover, the number of BrdU/DCX-positive cells increased with KRG administration. These results indicate that KRG administration increases neurogenesis and the differentiation of neuroblasts in the hippocampus and that the increased neuroblasts would affect learning and memory abilities in mice.

It is well known that ginsenosides enhance neurogenesis. Total saponins in ginseng enhance neurogenesis in a stroke animal model (Zheng et al., 2011). Moreover, ginsenoside Rd increased the proliferation of neuroblasts in the hippocampus of lead-exposed rats (Wang et al., 2013) and normal rats (Lin et al., 2012), while ginsenoside Rg1 was found to promote the cell proliferation in the hippocampus of adult mice (Shen and Zhang, 2004). Interestingly, Sun ginseng, a kind of dried and heated ginseng, has been shown to enhance neurogenesis in the hippocampus by promoting the activation of Akt and extracellular signal-regulated kinase (ERK) in the hippocampus of mice (Lee et al., 2013), while ginsenoside Rd was found to enhance neurite outgrowth of PC12 cells (Wu et al., 2016) and neurogenesis in cerebral ischemia-induced rat brains (Liu et al., 2015) via activation of Akt and ERK. KRG contains various ginsenosides including Rd and Rg1; therefore, these ginsenosides may play an important role in the increase of KRG-induced neurogenesis through PI3K/Akt and ERK-dependent pathways.

Neurotrophic and growth factors have emerged as crucial regulators of adult neurogenesis (Zhao et al., 2008); therefore, increasing neurotrophic factors can be a useful method for enhancing neurogenesis and improving impaired memory. In this study, CDNF and CNTF mRNA levels in the hippocampus were significantly increased by KRG. CDNF is known to protect and restore dopaminergic neurons in a Parkinson's disease animal model, and CDNF injection in the hippocampus has been shown to promote long-term memory in Alzheimer's disease mouse models and in wild-type mice (Kemppainen et al., 2015). CNTF plays a crucial role in nervous system maintenance and development by inducing neuronal survival and differentiation (Pasquin et al., 2015). Additionally, CNTF enhances memory function and neurogenesis in the hippocampus of normal adult C57BL/6 mice (Chohan et al., 2011). Interestingly, recent researches have shown that CDNF and CNTF promote PI3K-Akt signaling pathway. Four hours after intrastriatal injection of CDNF, PI3K-Akt pathway was activated in the striatum of normal rats (Voutilainen et al., 2017). CNTF enhances the migration of corneal epithelial stem cells by Akt phosphorylation (Chen et al., 2016) and intranasal injection of CNTF activates PI3K-Akt pathway in the brain (Alcala-Barraza et al., 2010). CDNF and CNTF do not activate ERK pathway in the brain (Purser et al., 2013; Voutilainen et al., 2017). Taken together, the results of this study indicate that KRG treatment enhances neurotrophic factors such as CDNF and CNTF in the hippocampus, which may enhance learning and memory as well as hippocampal neurogenesis through promoting PI3K-Akt pathway.

Ginseng has been traditionally used for a general restorative for human health as well as a therapeutic agent for treating diverse conditions in both males and females. However, there is a possibility that the effect of KRG varies depending on gender. Ginsenosides including Rb1, Bg1 and Rh1 have estrogenic activity (Park et al., 2017) and Daikenchuto, a Kampo formula including ginseng, caused more pronounced changes in gut microbiota in female than in male mice (Miyoshi et al., 2018). Since sex difference in the effects of ginseng is elusive (West and Krychman, 2015), it is necessary to study whether the response to ginseng is different between males and females.

In addition to this, there are some limitations in this study. First, the correlation between neurogenesis and motor function was not investigated. Second, the active compound in KRG was not studied. To clarify these, further studies will be conducted.

In summary, KRG administration can promote hippocampal neurogenesis as well as learning and memory abilities in mice, indicating that KRG has the potential for use in modulation of brain plasticity and memory function in patients with cognitive impairment.

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