Protective effect of Ginseng Radix on the interference of cell proliferation induced by maternal separation in the dentate gyrus of rat pups

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Abstract: Ginseng Radix (GR) is the root of Panax ginseng C. A. Meyer (Araliaceae). GR has been used for palpitations with anxiety, insomnia, forgetfulness, and restlessness from Deficient Qi and Blood.

Maternal separation (MS) in early life is very stressful for the neonate. It causes perturbation of neuroendocrine and physiological conditions, and increases vulnerability for psychiatric diseases and neuronal degeneration. In order to investigate the protective effects of GR on the interference of cell proliferation induced by MS in the dentate gyrus (DG), we administered it to rat pups with MS. Rat pups (outbred SD strain) were separated from their mothers on postnatal day 14 and treated with GR (100 mg/kg) and 5-bromo-2'-deoxyuridine (BrdU) (50 mg/kg) for 7 days, after which BrdU-specific immunohistochemistry was carried out. In the separation group, the number of BrdU-positive cells in the DG was significantly decreased than in control (p < 0.05). In contrast, in the ginseng-treated group with MS, the number of BrdU-positive cells in the DG was significantly increased than in the separation group, as expected (p < 0.05). These results indicate that GR protects the interference of cell proliferation induced by MS, and we suggest that GR may be useful in the treatment of the diseases associated with MS.

Keywords: Ginseng Radix, dentate gyrus, hippocampus, maternal separation, 5-bromo-2-deoxyuridine, BrdU

1. Introduction

Ginseng Radix (GR), the root of Panax ginseng C. A. Meyer (Araliaceae), is one of the most popular tonics that has been exploited in Korea, China, Japan, and other Asian countries. GR, that is called In-Sam in Korea, has been used as a herbal medicine for enhancing various therapeutic applications for a long time.

The first record of GR prescription as a medicinal herb appeared in 'Shin-Nong-Bon-Cho-Gyoun' which was originated from the ancient China. Property and flavor of GR are slightly warm, non-toxic, sweet, and slightly bitter. It acts on the Spleen, Lung, and Heart channels. According to 'Shin-Nong-Bon-Cho-Gyoun', GR is not only the primary medicine administered for promoting vitality of the five viscera (Liver, Heart, Lung, Kidney, and Spleen), but also the therapeutic medicine that helps restore composure, or remove causes of illness of the five viscera. And GR improves eyesight and increases longevity if man would take it for a long period of time.1-3)

Ginseng has been reported to have various pharmacological effects in the central nervous system, such as memory facilitation4-5) and behaviors.6) In addition, it has anti-oxidant
activity, anxiolytic activity, anti-fatigue effect, anti-obese effect, anti-carcinogenic effect, anti-stress effect, and adaptogenic effects on the acute hypothermia. GR contains many active ingredients, e.g. ginsenosides that has beneficial neuroprotective effects. This herb stimulates an immune function in the elderly, and has a potent recovery of impaired brain growth exposed to ethanol in neonatal rats. Neonates instinctively require the maternal care (MC) to survive in the normal environment. Mother supports neonates with sources of nutrition and warmth for the behavioral developments. MC has influence on the maintenance of a normal growth, physiological maturation, and the development of neural systems. Maternal separation (MS) initiates complex responses involving both physiological and behavioral changes. Several investigators have suggested that MS in early life increases vulnerability for neuropsychiatric diseases such as depression, personality disorders, and anxiety disorders. In addition, MS interferes with normal brain development by increasing cell death of neurons and glia in the infant rat brain.

The granule cell population of the dentate gyrus (DG) is produced dominantly during postnatal period in rats. It has been proved that process of neurogenesis, the development of new neurons, occurs in the DG in rodents and human. Neurogenesis in the DG is mediated by stress, complexity of circumstances, hormones, and pharmacological actions (i.e., NMDA receptor activation).

However, the study about the effect of GR acts on the cell proliferation in maternally-separated rat pups, has not been published up to date. In the present study, the protective function of GR on the interference of cell proliferation induced by MS in the DG were investigated via 5-bromo-2'-deoxyuridine (BrdU)-specific immunohistochemistry.

2. Materials and methods

1. Animals

Sprague-Dawley rat pups of the postnatal day (pnd) 9 were purchased from a commercial breeder (Samtako, Kyung-gi, Korea). On arrival at the laboratory, the pups with two dams (12 and 12 pups from each) were housed under controlled temperature (22°C) and 12/12h (light/dark) cycle conditions with food and water made available ad libitum. The experimental procedures were maintained under the animal care guidelines of the National Institutes of Health.

2. Drug preparation

GR, the roots of 6-year-old "white ginseng", was purchased from Kum-San drug market in Korea. It was identified at the Department of Oriental Pharmacy, College of Pharmacy, Woosuk University.

To gain extracts of GR, dried GR (200 g) were added to distilled water (1 L), and heat-extracted twice for 8 h at 75°C. The filtrates were united, evaporated with a rotary vacuum evaporator, and lyophilized. The weight of resulting powder was 32.3 g. The extract was stored at -20°C till use.

3. Experimental procedure

We used a procedure for mother-infant separation that has been observed by Lee et al. to result in neuronal differences between MS and control offsprings. The pups and dams were monitored until pnd 13. On pnd 14, the pups were designated to four groups, with six pups in each group (see Fig. 1).

From pnd 14 until pnd 20, all the rat pups were injected i.p. with BrdU (50 mg/kg, Sigma, MO, USA) after saline and GR administrations. BrdU was dissolved in 0.9% NaCl and filtered at 0.45 μm. All treatments were carried out for 7 consecutive days.

4. Tissue preparation

On pnd 21, rat pups were deeply anesthetized with Zoletil 50 (10 mg/kg, i.p.; Vibac, Carros, France) and then transcardially perfused with 0.05 M phosphate buffer saline (PBS, pH 7.4), and fixed by chilled 4% paraformaldehyde (Sigma, MO, USA) in 0.1 M phosphate buffer (PB, pH 7.4). After perfusion, all brains were removed and postfixed in the same fixative solution for 1 day at 4°C and then transferred into 30% sucrose solution for cryoprotection. Coronal
7 14 21 (pnd)

A

B

C

D

Maternal care condition
Maternal separation

Fig. 1 Schematic representation of experimental procedure.

A: saline-treated maternal care (MC) group; Control group: pups were bred under normal, MC conditions, and saline was administered intraperitoneally (i.p.) once a day.

B: ginseng-treated MC group; Con+ginseng group: pups were bred under normal, MC conditions, and Ginseng Radix (GR, 100 mg/kg) was administered i.p. once a day.

C: saline-treated maternal separation (MS) group; Separation group: pups were separated from their mother and littermates, i.e., each was placed in a separate, single cage, and saline was administered i.p. once a day.

D: ginseng-treated MS group; Sep+ginseng group: pups were separated from their mother and littermates, i.e., each was placed in a separate, single cage, and GR (100 mg/kg) was administered i.p. once a day.

Abbreviation: pnd = postnatal day

sections of the brains were cut (40 μm in thickness) using a freezing microtome (Leica, Nußloch, Germany).

5. BrdU immunohistochemistry

For detection of newly generated cells in the DG, free-floating sections were processed for BrdU-specific immunohistochemistry as described previously.25) Sections were permeabilized by incubation in 0.5% Triton X-100 (Sigma, MO, USA), followed by incubation in 40% formamide (Sigma, MO, USA)-2X standard saline citrate (Sigma, MO, USA) at 65°C and subsequently denatured in 2 N HCl at 37°C and then neutralized in 0.1 M sodium borate (pH 8.5, Sigma, MO, USA). After washing in 50 mM PBS, the sections were incubated with BrdU-specific mouse monoclonal antibody (1:600; Boehringer Mannheim, Germany). The sections were rinsed and stained according to the horseradish peroxidase method (Elite ABC system, with biotinylated horse anti-mouse antibodies and diaminobenzidine as chromogen; Vector Laboratories, Burlingame, CA, USA). For immunostaining, the sections were reacted with 0.02% 3,3’-diaminobenzidine (Sigma, MO, USA) containing nickel chloride (40 mg/ml) and 0.03% hydrogen peroxide in 0.05 M Tris-HCl (pH 7.6, Sigma, MO, USA). The sections were mounted onto gelatin-coated slides, air-dried, and counterstained with aqueous hematoxylin. Finally, the sections were dehydrated in ethanol, cleared in xylene, and coverslipped. The number of BrdU-positive cells in the granule cell layer through the DG was counted hemilaterally in each of the selected sections using an Olympus microscope (Olympus, Japan).

6. Statistical analysis

The data were analyzed by one-way ANOVA using the statistical software SPSS (version 7.5). Specific comparisons were made with Tukey HSD's post-hoc test.

3. Results

It was presumed that GR might have positive effects on cell proliferation during MS. To investigate the protective effect of GR on the reduction of cell proliferation during MS, we administered GR (i.p., 100 mg/kg) to maternally-separated rat pups.

Daily body weight changes in each group were measured for consecutive 7 days. Control and the ginseng-treated MC group were showed a tendency to increase compared to that of the separation group. Weight loses for 3 days, from 2nd day to 4th day of experiment, were also measured. In the separation group, body weight was significantly decreased compared to that of control and the ginseng-treated MC group (p < 0.05) (see Fig. 2). In the ginseng-treated MS group, body weight changes did not increase compared to that of the separation group.
Fig. 2 Daily body weight change for consecutive 7 days.
Saline-treated pups with MC (Control); ginseng-treated (100 mg/kg) pups with MC (Con+ginseng); saline-treated pups with MS (Separation); ginseng-treated (100 mg/kg) pups with MS (Sep+ginseng). The data are presented as mean ± SEM. *p < 0.05, both Separation and Sep+ginseng were significantly different from Control and Con+ginseng.

MS interferes cell proliferation in the DG; in contrast, GR enhances cell proliferation in the same region of MS group. In other words, the number of BrdU-positive cells in the DG of the ginseng-treated MS group was increased compared to that of the separation group (see Fig. 3).

In the separation group, the number of BrdU-positive cells in the DG was significantly decreased compared with control group (p < 0.05). In contrast, in the ginseng-treated MS group, the number of BrdU-positive cells in the DG was significantly increased than in the separation group, as expected (p < 0.05) (see Table 1). These results indicated that GR could protect the interference of cell proliferation induced by MS in the DG of rat pups.

Fig. 3 Bright-field photomicrographs showing BrdU-positive cells in the dentate gyrus.
BrdU-specific immunohistochemistry was done in sections obtained 2 day after the last day of the injections from each group; saline-treated pups with MC (A, a); ginseng-treated (100 mg/kg) pups with MC (B, b); saline-treated pups with MS (C, c); ginseng-treated (100 mg/kg) pups with MS (D, d). All treatments were given for consecutive 7 days after pnd 14. Scale bar represents 400 μm (A, B, C, D), and 40 μm (a, b, c, d), respectively.

Table 1. BrdU-positive cells in the dentate gyrus

<table>
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<tr>
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<th>Maternal care</th>
<th>Maternal separation</th>
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<tr>
<td>Saline-treated</td>
<td>188 ± 11</td>
<td>95 ± 11*</td>
</tr>
<tr>
<td>Ginseng-treated</td>
<td>201 ± 28</td>
<td>168 ± 13</td>
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Saline-treated pups with MC (Control); ginseng-treated (100 mg/kg) pups with MC (Con+ginseng); saline-treated pups with MS (Separation); ginseng-treated (100 mg/kg) pups with MS (Sep+ginseng). The data are presented as mean ± SEM. Differences between groups were assessed with one-way ANOVA with Tukey
HSD's post-hoc test. *p < 0.05, compared with Control, Con+ginseng, and Sep+ginseng.

4. Discussion

The aim of this study was to investigate the protective effect of GR on the interference of cell proliferation induced by MS at the developmental stage of rat pups in brain.

Alternative medicines such as herbal products are being used for therapeutic purposes. GR is the most popular herbal medicine that commonly used. Clinically, GR is used for palpitations with anxiety, insomnia, forgetfulness, and restlessness from Deficient Qi and Blood. In addition, it cures many diseases such as shallow respiration, shortness of breath, cold limbs, lack of appetite, chest and abdominal distension, and profuse sweating.32) Recent evidences suggest that administration of GR can improve cognitive performance in animals and humans.15,33) GR enhanced cell proliferation in DG of rats with streptozotocin-induced diabetes.24)

Recently, Lee et al.25) reported that MS consecutive 7 days after pnd 14 inhibited cell proliferation in the DG. It's well established that the early loss of MC has influence on the vulnerability of the infant to psychiatric disorders, such as depression and anxiety during the life span.21-23) In addition, a disturbance of mother-infant relationship can lead to anxious symptoms and personality disorders.35) Naturally occurring variations of maternal behaviors (licking/grooming) can influence hippocampal development in the rat.36) It is reported that neonatal MS resulted in reduction of hippocampal mossy fiber density in adult rat.37) Interestingly, MS increased expression of Nerve Growth Factor (NGF) mRNA in the DG and the hilus of the hippocampus, and NGF is a neurotrophin involved in growth and differentiation of central cholinergic neurons.31) Previous studies have demonstrated that early environmental event, i.e. MS, appears persistently to alter glucocorticoids feedback mechanisms under stressful situations, and thus it enhances hypothalamic-pituitary-adrenal (HPA) responses to stress. Furthermore, it seems that variations of HPA functioning may affect cognitive and emotional processes.38,39)

Acupuncture at acupoint Shenmen (HT7) appeared to stimulate cell proliferation of the DG in maternally-separated rat pups.40) In addition, fluoxetine, a drug for depression, had been invented that it enhanced cell proliferation and prevented apoptosis in DG of maternally-separated rats.25)

To be convinced of newly generated cells in the DG, Brdu-specific immunohistochemistry is used for this study. DNA synthesis by subpopulations of cells can be tracked by making use of radioactive form thymidine, but it can also be done by using of its analog Brdu. In the newly synthesized DNA thymidine is partly replaced by Brdu.

The present study demonstrates that MS during consecutive 7 days after pnd 14 decreases cell proliferation in the DG of rat pups. Early MS is one of the models for environmentally-mediated brain plasticity. Ginseng treatment appeared to protect the interference of cell proliferation induced by MS in the DG. Therefore, GR may provide new therapeutic application to MS. Further researches will be required to explain the protective mechanisms of GR on neuronal injuries.

5. Conclusion

In conclusion, increased cell proliferation appears to occur in concert with ongoing neuropsychiatric damages after MS to infant rats. Thus, early MS has influence on hippocampal structure and function, partially. This results showed that MS after pnd 14 decreased the newly generated cells in the DG, in contrast, administration of GR (100 mg/kg) protected the interference of cell proliferation induced by MS in the DG.

Acknowledgment

This study was supported by a grant of the Oriental Medicine R&D Project, Ministry of Health & Welfare, Republic of Korea (00-PJ9-PG1-CO04-0002).

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