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원 저

인삼이 흰쥐의 치상회에서 알코올에 의한 새로운 신경세포 생성 및 nitric oxide synthase 발현에 미치는 영향

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Effects of *Ginseng radix* on Alcohol-induced Decrease in New Cell Formation and Nitric Oxide Synthase Expression in Dentate Gyrus of Rats

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목적 : 본 연구에서는 알코올 독성에 대하여 흰쥐의 치상회에서 새로운 신경세포의 생성 및 nitric oxide synthase (NOS) 발현에 인삼이 미치는 영향을 5-bromo-2-deoxyuridine (BrdU) 면역조직화학법 및 nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) 조직화학법을 통해서 관찰하고자 한다.

방법 : 실험동물을 정상군, 인삼처치군, 알코올처치군 및 알코올-인삼 처치군으로 분류하여 각각의 실험군에 3일간 BrdU (50mg/kg)를 복강주사하였다. 인삼처치군은 30 mg/kg 용량의 인삼 전탕액을 중완혈에 약침주사하였고, 알코올 처치군은 2 g/kg 용량의 알코올을 투여하였으며, 알코올-인삼 처치군은 2 g/kg 용량의 알코올 및 30mg/kg 용량의 인삼 전탕액을 투여한 후 각각의 BrdU 양성 세포수와 NADPH-d 양성세포수를 관찰하였다.

결과 : 알코올 투여군은 BrdU 양성세포 및 NADPH-d 양성세포 발현이 감소하였으나, 인삼 및 알코올-인삼처치군에서는 알코올 투여군에 비해서 모두 증가하였다.

결론 : 인삼은 알코올에 의해서 유발된 새로운 신경세포 생성의 감소에 대하여 보호효과가 있으며, 알코올에 의해서 부가적으로 영향받는 산화질소는 세포생성 조절에 중요한 역할을 하는 것으로 사려된다. *(J Korean Oriental Med 2002;23(3):26-32)*

Key Words: *Ginseng radix*, Alcohol, 5-bromo-2-deoxyuridine, Nicotinamide adenine dinucleotide phosphate-diaphorase, Nitric oxide synthase.

INTRODUCTION

Ginseng radix, the root of Panax ginseng C.A. Meyer (Araliaceae), is one of the most famous Oriental medical herbs and has several therapeutic applications. It is well documented that *Ginseng radix* possesses a number of pharmacological effects including

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hypotensive, cardiotonic, sedative, aphrodisiac, antiaging, and antioxidant actions[1-3]. Traditionally, *Ginseng radix* has been widely used in the treatment of alcohol-related problems[4].

Ethanol (EtOH) abuse is known to cause substantial neuronal loss in several regions of the brain, one of the major targets of the toxic effects of EtOH. EtOH induces death in a variety of cells including astroglia[5] and neuroblastoma cells[6] *in vitro*, and that it triggers apoptotic neurodegeneration in the developing rat brain in vivo[7]. In addition, EtOH intake during the developmental stage has been associated with deficits in learning and memory[8].

The hippocampal formation plays a central role in learning and memory formation[9]. It has been demonstrated that the process of neurogenesis, the birth of new neurons, occurs in the hippocampal dentate gyrus in a variety of mammals, including humans[10]. In previous studies, several factors, including glucocorticoids, estrogen, N-methyl-D-aspartate receptor antagonists, serotonin, ischemia, seizures, and various environmental stimuli have been shown to influence the proliferation of granule cell precursors and/or neurogenesis in the adult dentate gyrus[11-14].

Nitric oxide (NO), endogenously generated from L-arginine by NO synthase (NOS), is a free radical with signaling functions in the central nervous system (CNS). It has been implicated in numerous physiological and pathological processes in the brain15). Nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) is a histochemical marker specific for NOS in the CNS. Neurons containing NADPH-d have been reported to be relatively resistant to various toxic insults and neurodegenerative disorders[16]. It has been shown that alcohol inhibits NO production in vivo, and thus it may be suggested that NO is of relevance in the pathogenesis of alcohol-induced brain damage[17]. Ogura et al.[18] suggested that the signal transduction mechanism of neuronal cell differentiation which is involved by trans-retinoic acid appears to be distinct from the NO-mediated pathway. And it was suggested that NOS may play an important role during neurogenesis in the subventricular zone of adult mice[19].

It has not yet been established whether *Ginseng radix* exerts a protective effect on alcohol-induced decrease in cell proliferation and NOS expression. In the present study, the effects of *Ginseng radix* on cell proliferation and NOS expression in the dentate gyrus of acutely alcohol-intoxicated rats were investigated via BrdU imunohistochemmistry and NADPH-d histochemistry.

Materials and Methods

1. Animals and treatment

Male Sprague-Dawley rats weighing 150 ± 10g (5 weeks in age) were used in the present study. The experimental procedures were performed in accordance with the guidelines of the NIH and the Korean Academy of Medical Sciences. Each animal was housed at a controlled temperature (20 ± 2°C) and maintained under light-dark cycles consisting of 12 h of light and 12 h of darkness (lights on from 07:00 h to 19:00 h), with food and water made available ad libitum. Animals were divided into four groups: the control group, the *Ginseng radix*-treated group, the alcohol-treated group, and the alcohol- and *Ginseng radix*-treated group (n = 5 for each group). Rats of the control group were injected intraperitoneally with 5-bromo-2-deoxyuridine (BrdU) (50 mg/kg; Sigma Chemical Co., St. Louis, MO, USA) for 3 consecutive days, while animals of the *Ginseng radix*-treated group were injected with an equivalent dose of BrdU and 30 mg/kg of *Ginseng radix* extracts for the same duration of time. In the alcohol-treated group, each animal was injected with 50 mg/kg of BrdU and 2 g/kg of alcohol

for 3 days, and animals of the *Ginseng radix*- and alcohol-treated group received injections of BrdU, alcohol, and *Ginseng radix* extracts in doses used on animals of other groups.

2. Preparation of the aqueous extracts of Ginseng radix

To obtain aqueous extracts of *Ginseng radix*, 200 g of Ginseng radix was added to distilled water, heat-extracted, pressure-filtered, concentrated with a rotary evaporator and lyophilized. The resulting powder, weighing 25 g (a collection rate of 12 %) was diluted to the concentration needed with a saline solution and filtered through a 0.45 ㎛ syringe filter before use.

3. Blood alcohol concentration measurements

For analysis of serum alcohol concentration, blood was collected from animals via cardiac puncture 2 h after the last injection, and the blood alcohol concentration was measured using a Sigma Diagnostics kit (Sigma Chemical CO., St. Louis, MO, USA) according to the manufacturers protocol.

4. Tissue preparation

For the sacrificial process, animals were first fully anesthetized with ZoletilR (10 mg/kg, i.p.; Vibac, Carros, France), then transcardially perfused with 50 mM phosphate-buffered saline (PBS), and then fixed with a freshly prepared solution consisting of 4% paraformaldehyde in 100 mM phosphate buffer (PB, pH 7.4). The brains were then removed, postfixed in the same fixative overnight, and transferred into a 30% sucrose solution for cryoprotection. Coronal sections of 40 ㎛ thickness were made with a freezing microtome (Leica, Nussloch, Germany).

5. BrdU immunohistochemistry

For detection of newly generated cells in the dentate gyrus, the associated BrdU incorporation was visualized via a previously described immunohistochemical method[10,13]. First, eight sections on average were collected from each brain within the dorsal hippocampal region spanning from Bregma -3.30 mm to -4.16 mm. Sections were permeabilized by incubation in 0.5% Triton X-100 in PBS for 20 min, then pretreated in 50% formamide-2 x standard saline citrate (SSC) at 65℃ for 2 h, denaturated in 2 N HCl at 37℃ for 30 min, and rinsed twice in 100 mM sodium borate (pH 8.5). Afterwards, the sections were incubated overnight at 4 ℃ with a BrdU-specific mouse monoclonal antibody (1:600; Boehringer Mannheim, Mannheim, Germany). The sections were washed three times with PBS and incubated for 1 h with a biotinylated mouse secondary antibody (1:200; Vector Laboratories, Burlingame, CA, USA). Then, the sections were incubated for another 1 h with VECTASTAIN Elite ABC Kit (1:100; Vector Laboratories, Burlingame, CA, USA).

For visualization, the sections were incubated in 0.02% 3,3-diaminobenzidine (DAB) containing nickel chloride (40 mg/ml) and 0.03% hydrogen peroxide in 50 mM Tris-HCl (pH 7.6) for 5 min. Following BrdU-specific staining, dual immunostaining was performed on the same sections using a mouse anti-neuronal nuclei antibody (1;300; Chemicon International, Temecula, CA, USA). Following incubation with the said antibody, the sections were washed three times with PBS, incubated for 1 h with a biotinylated mouse secondary antibody, and processed with VECTASTAIN ABC Kit. For visualization, the sections were incubated in 0.02% DAB (40 mg/ml) and 0.03% hydrogen peroxide in 50 mM Tris-HCl (pH 7.6) for 5 min. The sections were then washed with PBS and mounted onto gelatin-coated slides. The slides were dried and coverslips were mounted using Permount.

6. NADPH-d histochemistry

For NADPH-d activity, sections were stained according to a previously described protocol19). In brief, free-floating sections were incubated at 37℃ for 60 min in 100 mM PB containing 0.3 % Triton X-100, 0.1 mg/ml nitroblue tetrazolium, and 0.1 mg/ml β-NADPH. The sections were then washed three times with PBS and mounted onto gelatine-coated slides. The slides were air-dried overnight at room temperature, and coverslips were mounted using PermountR.

7. Data analyses

The area of the dentate gyrus region was measured hemilaterally in each of the selected sections using an image analyzer (Multiscan, Fullerton, CA, USA). The total numbers of BrdU-positive and NADPH-d-positive cells were obtained and the results were expressed as number of cells per mm2 of cross-sectional area of the granular layer of the dentate gyrus. Statistical differences were determined by one-way analysis of variance (ANOVA) followed by Scheffe's post-hoc analysis, and results were expressed as mean ± S.E.M. Differences were considered significant for $P < 0.05$.

Results

1. Blood alcohol concentration

The serum alcohol concentration was 70.94 ± 3.62 mg/dl in the alcohol-treated groups and 0 or negligible in the saline-treated group.

2. Number of BrdU-positive cells in each group

The number of BrdU-positive cells in the dentate gyrus was about 287.44 ± 15.20/mm² in the control group, 311.56 ± 17.44/mm² in the Ginseng radix-treated group, 166.32 ± 18.56/mm² in the alcohol-treated group, and 293.84 ± 21.44/mm² in the alcohol- and Ginseng radix-treated group (Fig. 2).

3. Number of NADPH-d positive cells in each group

The number of NADPH-d-positive cells in the dentate gyrus was about 176.92 ± 6.16/mm² in the control group, 207.72 ± 10.80/mm² in the *Ginseng radix*-treated group, 119.08 ± 7.28/mm² in the alcohol-

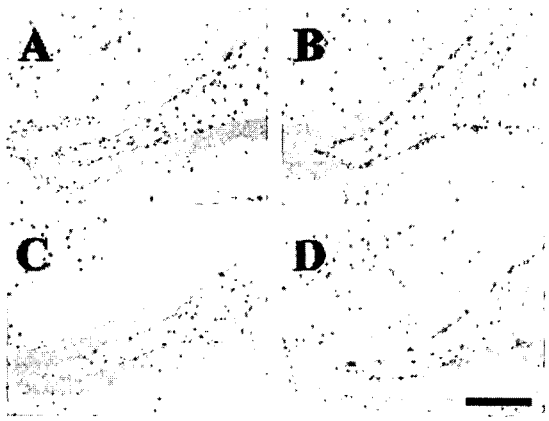

Fig. 1. Photomicrograph of BrdU-positive cells. Sections were stained for BrdU and NeuN. A; Control group, B; *Ginseng radix*-treated group, C; Alcohol-treated group, D; Alcohol- and *Ginseng radix*-treated group. Scale bar represents 100 μm.

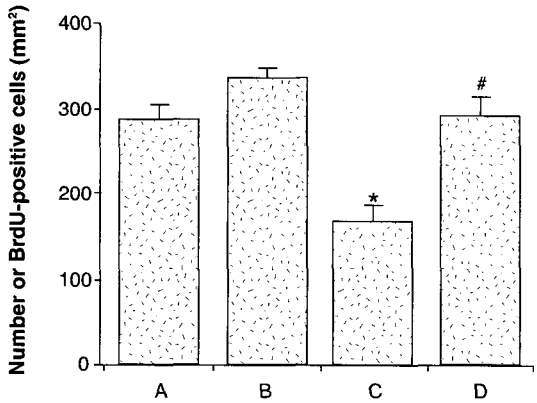

Fig. 2. Mean number of BrdU-positive cells in the subgranular layer of the dentate gyrus in each group. A; Control group, B; *Ginseng radix*-treated group, C; Alcohol-treated group, D; Alcohol- and *Ginseng radix*-treated group. * represents $P < 0.05$ compared to the control group. # represents $P < 0.05$ compared to the alcohol-treated group.

treated group, and 205.60 ± 7.72/mm² in the alcohol- and *Ginseng radix*-treated group (Fig. 4).

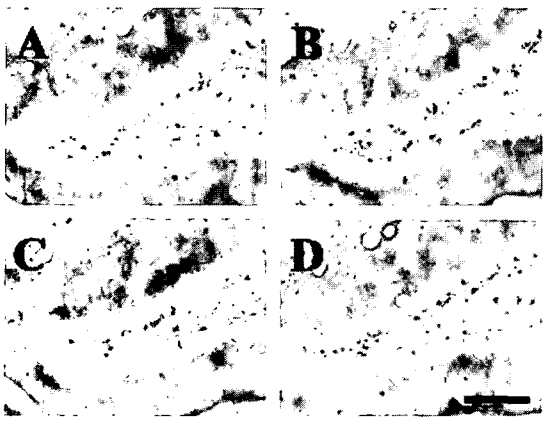

Fig. 3. Photomicrograph of NADPH-d-positive cells. Sections were stained for NOS. A; Control group, B; *Ginseng radix*-treated group, C; Alcohol-treated group, D; Alcohol- and *Ginseng radix*-treated group. Scale bar represents 100 ㎛.

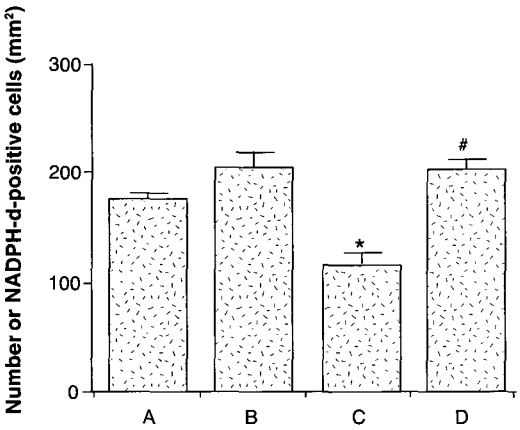

Fig. 4. Mean number of NADPH-d-positive cells in the subgranular layer of the dentate gyrus in each group. A; Control group, B; *Ginseng radix*-treated group, C; Alcohol-treated group, D; Alcohol- and *Ginseng radix*-treated group. * represents $P < 0.05$ compared to the control group. # represents $P < 0.05$ compared to the alcohol-treated group.

Discussion

In the present study, it was demonstrated that Ginseng radix increases the number of BrdU-positive and NADPH-d-positive cells with statistical insignificance under normal conditions, while these numbers were decreased significantly by alcohol administration. Interestingly, it was found that Ginseng radix extracts administration markedly increased number of reduced BrdU-positive and NADPH-d-positive cells by alcohol.

In variety of studies, the role of alcohol as an inducer of cell death has been described in hepatocytes and thymocytes[20,21]. Recently, it was also reported that alcohol induces apoptosis in several organs[5,6]. Particularly, Ikonomidou et al.[7] demonstrated that alcohol induces apoptosis in the developing rat brain. However, no report to date has reported on the effect of alcohol on cell proliferation in the dentate gyrus of hippocampus.

In the adult rat, neuronal precursors are known to reside in the subgranular zone of the dentate gyrus, where they proliferate and migrate continuously into the granule cell layer and differentiate into mature neurons, demonstrating the morphological and biochemical features of the surrounding neurons[11]. The alcohol-induced inhibition of new cell formation in the dentate gyrus seen in the present results could lead to a reduction in the formation of new granule neurons.

NO has been reported to participate in the formation of new neurons after birth[19], and it has been implicated as an intra- and inter-cellular signal that mediates neural development, neural plasticity, and alterations of synaptic function such as long-term potentiation[22]. Holscher et al.[23] suggested that neuron-specific NOS inhibitor has produced contradictory result in learning and memory in the rat.

Ginseng radix has been used in traditionally for several therapeutic applications, including alcohol intoxication. Initially, it was proposed that Ginseng radix accelerated alcohol metabolism and lowered blood alcohol levels by increasing ADH activity and plasma clearance[4,24]. A more recent study demonstrated that administration of Red Ginseng extracts to rats altered alcohol absorption from the gastrointestinal tract[25]. In the present study, Ginseng radix treatment was shown to increase both new cell formation and NOS expression in the dentate gyrus of alcohol-intoxicated rats.

Based on the results, it was demonstrated that aqueous extracts of Ginseng radix exert protective effect against alcohol-induced decrease in new cell formation, and it is possible that NO, which is affected adversely by alcohol, plays an important role in the regulation of cell proliferation.

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