Effect of *Liuweidihuang-tang* on Alcohol-induced Decrease in New Cell Formation in Rat Dentate Gyrus

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Liuweidihuang-tang has been traditionally used for the treatment of delayed mental and physical development in children, complications of diabetes, and glomerulonephritis. In the present study, the effect of *Liuweidihuang-tang* on cell proliferation in the dentate gyrus of alcohol-intoxicated Sprague-Dawley rats was investigated via 5-bromo-2' -deoxyuridine (BrdU) immunohistochemistry. Alcohol administration was shown to decrease the number of BrdU-positive cells in the dentate gyrus, while *Liuweidihuang-tang* treatment increased new cell formation in the dentate gyrus under normal conditions and alcohol intoxication. It was thus demonstrated that aqueous extract of *Liuweidihuang-tang* exerts a protective effect against alcohol-induced decrease in new cell formation.

Key words : *Liuweidihuang-tang*, alcohol, 5-bromo-2'-deoxyuridine, dentate gyrus, immunohistochenmistry, cell proliferation

Introduction

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 has been shown to induce death in a variety of cells including astroglia[1)] and neuroblastoma cells[2)] *in vitro* and to trigger apoptotic neurodegeneration in the developing rat brain *in vivo*[3)]. In addition, EtOH intake during the developmental stage has been associated with deficits in learning and memory[4)].

It is generally accepted that the hippocampus is involved in learning and memory[5,6)]. 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[7)]. 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[8-11)]. It has also been reported that newly generated cells mature into functional neurons in the adult mammalian brain[6)].

Liuweidihuang-tang, an oriental herbal medicinal formulation, has been traditionally used for the treatment of delayed mental and physical development in children, complications of diabetes, and glomerulonephritis[12,13)]. However the effects of *Liuweidihuang-tang* on alcohol-induced suppression of new cell formation have not studied yet. In the present study, the protective effect of *Liuweidihuang-tang* on alcohol-induced suppression of cell proliferation was studied using the 5-bromo-2' -deoxyuridine (BrdU) immunohistochemistry.

Materials and Methods

1. Animals and treatment

Male Sprague-Dawley rats weighing 120 ± 10g (4 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 six groups: the control group, the alcohol-treated group, the alcohol- and 5 mg/kg *Liuweidihuang-tang*-treated

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group, the alcohol- and 10 mg/kg *Liuweidihuang-tang*-treated group, the alcohol- and 50 mg/kg *Liuweidihuang-tang*-treated group, and the alcohol- and 100 mg/kg *Liuweidihuang-tang* -treated group (n = 5 for each group). Rats of the control group were injected intraperitoneally with BrdU (50 mg/kg; Sigma Chemical Co., St. Louis, MO, USA) once a day for 3 consecutive days, while animals of the alcohol-treated group were injected with 50 mg/kg BrdU and 2 g/kg alcohol once a day over the same duration of the time. Animals of the alcohol- and *Liuweidihuang-tang*-treated groups were injected with BrdU and alcohol in doses used on animals of the other groups and with *Liuweidihuang-tang* extracts at the respective doses once a day for 3 days.

2. Preparation of *Liuweidihuang-tang*

The ingredients of *Liuweidihuang-tang* are as follows: Rehmanniae Radix 16g, Dioscorae Radix 8g, Corni Fructus 8g, Alimatis Rhizoma 6g, Moutan Cortex Radicis 6g, Hoelen 6g, Maximowicziae Fructus 8g and Cervi Cornu 4g. All ingredients were obtained from the Kyung Dong marketplace (Seoul, Korea).

After washing, each component of *Liuweidihuang-tang* was immersed in cold water for 12 h. To obtain the aqueous extracts of *Liuweidihuang-tang*, the ingredients were added to distilled water, heat-extracted, pressure-filtered, concentrated with a rotary evaporator and lyophilized. The resulting powder, weighing 3.5 g, was diluted to the concentration needed with normal saline solution and filtered through a 0.45 mm syringe filter before use.

3. Plasma alcohol measurement

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 the Sigma Diagnostics® kit (Sigma Chemical Co., St. Louis, MO, USA) according to the manufacturer's protocol.

4. Tissue preparation

For the sacrificial process, animals were fully anesthetized with Zoletil® (10 mg/kg, i.p.; Vibac, Carros, France), 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 mm thickness were made with a freezing microtome (Leica, Nussloch, Germany).

5. BrdU immunohistochemistry

For detection of newly generated cells in the dentate gyrus, BrdU incorporation was visualized via a previously described immunohistochemical method14). First, eight sections on average were selected 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, 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). The sections were then incubated for another 1 h with VECTASTAIN® Elite ABC Kit (1:100; Vector Laboratories, Burlingame, CA, USA).

For visualization, the sections were incubated with 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 with 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. Data analysis

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 number of BrdU-positive cells was obtained using a previously described method14), and the results were expressed as number of cells per mm2 of cross-sectional area of the granular layer of the dentate gyrus.

7. Statistical analysis

Statistical differences were determined by one-way analysis of variance (ANOVA) followed by Scheffe' s post-hoc test, and results are expressed as mean ± S.E.M. Differences were considered significant for $P < 0.05$.

Results

The serum alcohol concentration was 105.91 ± 4.62 mg/dl in the alcohol-treated groups and 0 or negligible in the saline-treated group.

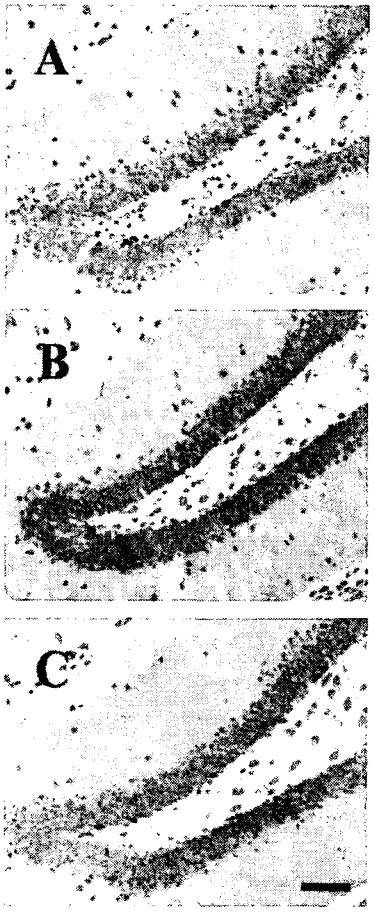

Fig. 1. Photomicrographs of BrdU-positive cells in the dentate gyrus of each group. A, Control group; B, Alcohol-treated group; C, Alcohol- and 100 mg/kg Liuweidihuang-tang -treated group. Sections were stained for BrdU (Black) and NeuN (brown). Scale bar represents 100 μm.

The number of BrdU-positive cells in the dentate gyrus was 112.88 ± 5.40/mm2 in the control group, 78.88±4.84/ mm2 in the alcohol-treated group, 112.66±6.12/ mm2 in the alcohol- and 5 mg/kg Liuweidihuang-tang-treated group, 114.00±3.59/ mm2 in the alcohol- and 10 mg/kg *Liuweidihuang- tang*-treated group, 149.60±6.25/ mm2 in the alcohol- and 50 mg/kg Liuweidihuang-tang-treated group, and 171.77±5.02/ mm2 in the alcohol- and 100 mg/kg *Liuweidihuang-tang*-treated group.

Alcohol administration markedly decreased the number of BrdU-positive-cells, and *Liuweidihuang-tang* treatment was shown to significantly increase the number of BrdU-positive-cells in the dentate gyrus dose-dependently in normal and alcohol-intoxicated rats.

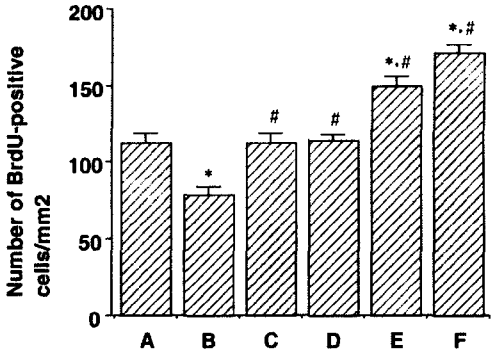

Fig. 2. Mean number of BrdU-positive cells in the subgranular layer of the dentate gyrus in each group. A, Control group; B, Alcohol-treated group; C, Alcohol- and 5 mg/kg Liuweidihuang-tang-treated group; D, Alcohol- and 10 mg/kg Liuweidihuang-tang-treated group; E, Alcohol- and 50 mg/kg Liuweidihuang-tang-treated group; F, Alcohol- and 100 mg/kg Liuweidihuang-tang-treated group. * represents P < 0.05 compared to the control group. # represents P < 0.05 compared to the alcohol-treated group.

Discussion

The protective effect of *Liuweidihuang-tang* against alcohol-induced suppression of cell proliferation was examined in the present study. In adult rats, neuronal precursors are known to reside in the subgranular zone (SVZ) of the dentate gyrus, where they proliferate and migrate continuously into the granule cell layer and differentiate into mature neurons during adult life, demonstrating the morphological and biochemical features of the surrounding neurons[7,8,15]. It has been suggested that these newly formed neurons are essential for the formation of memory[16,17].

In the present study, alcohol administration was shown to decrease the number of BrdU-positive-cells in the dentate gyrus. It is generally accepted that alcohol abuse results in numerous complications involving the central nervous system (CNS) such as alcoholic dementia, cognitive deficits, learning and memory deficits, and behavioral problems such as hyperactivity, impulsivity, poor socialization and communication skills[18-20]. In a previous study, alcohol administration was shown to decrease the number of BrdU-positive-cells in the dentate gyrus[14]. The alcohol-induced decrease in BrdU-positive cells in the dentate gyrus implies a reduction in newly formed granule neurons. Exposure to alcohol during neural development is known to lead to substantial neuronal loss in multiple brain regions[21]. Studies involving animal models have revealed the life-long sequelae of prenatal alcohol exposure including poor somatic growth, major organ malformation, craniofacial anomalies, and associated CNS dysfunction[22]. CNS dysfunction is expressed as reduced capacity for basic adaptive functions, including impaired neural plasticity, poor learning, and abnormal

response to challenging situation[23]. Alcohol was reported to enhance apoptosis[24,25].

In this study, it was demonstrated that the aqueou extract of *Liuweidihuang-tang* exerts a protective effect against alcohol-induced decrease in new cell formation. *Liuweidihuang-tang* has been reported to boost the activity of natural killer cells, macrophages, and cells of various T-lymphocyte subgroups[26,27]. *Liuweidihuang-tang* treatment is also said to be effective in the symptom improvement of proteinuria in diabetic nephropathy[13] and to improve the cardiac functions[28]. Alcohol injection inhibited the number of BrdU-positive cells, and *Liuweidihuang-tang* treatment increased the number of BrdU-positive cells in the dentate gyrus of the hippocampus dose-dependently, in this study. Based on the results of this study, it can be suggested that alcohol-induced memory impairments mau be due to alcohol-induced inhibition of new cell formation and that *Liuweidihuang-tang* may be of therapeutic utility increase cell proliferation property.

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