Puerariae radix increases Alcohol-induced Suppressed Cell Proliferation and Expression of Nitric Oxide Synthase in Dentate Gyrus of Rats

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<Abstract>

Traditionally, aqueous extracts of *Puerariae radix* had been used for the treatment of alcohol-related problems. In the present study, the effect of *Puerariae radix* on cell proliferation and expression of nitric oxide synthase (NOS) in the dentate gyrus of alcohol-intoxicated rats were investigated *via* 5-bromo-2'-deoxyuridine (BrdU) immunohistochemistry and nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) histochemistry, respectively. Sprague-Dawley rats weighing 150 ± 10 g were divided into four groups: the control group, the *Puerariae radix*-treated group, the alcohol-treated group, and the alcohol- and *Puerariae radix*-treated group. The numbers of both BrdU-positive and NADPH-d-positive cells in the dentate gyrus were inhibited significantly by alcohol administration, while *Puerariae radix* treatment was shown to increase those numbers. In this study, it was revealed that *Puerariae radix* possesses protective effect against alcohol-induced suppressed new cell formation and NOS expression in the dentate gyrus. Based on the results, it is possible that NO, which might play an important role in the regulation of cell proliferation, is a major target of the toxic effects of alcohol.

Key words : *Puerariae radix*, alcohol, dentate gyrus, 5-bromo-2'-deoxyuridine, nicotinamide adenine dinucleotide phosphate-diaphorase.

Introduction

Traditionally, *Puerariae radix* has been used as an antipyretic, antidiarrhetic, diaphoretic, and antiemetic agent. In Oriental medicine, medications based on *Puerariae radix* have been found to be useful in the treatment of alcohol-related problems, as an anti-intoxication and anti-drinking agent and in the treatment of various liver diseases caused by alcohol abuse[1]. Neurogenesis comprises cell proliferation, differentiation, and migration. 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[2,3]. Several factors, including glucocorticoids, estrogen, *N*-methyl-D-aspartate receptor, serotonin, ischemia, seizures, and environmental stimuli are known to influence the proliferation of granule cell precursors in the adult dentate gyrus[2-4]. Alcohol consumption is known to cause substantial neuronal loss in several regions of the brain[5-7]. It has been reported that alcohol 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]. Alcohol intake during the developmental stage has been correlated with deficits in learning and memory[8]. Nitric oxide (NO), synthesized from L-arginine by nitric oxide synthase (NOS), is a free radical molecule with signaling functions; it has been implicated in numerous physiological and pathological processes in the brain[9]. 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[10]. Moreno-López et al[11] suggested that NOS may play an important role during neurogenesis in the subventricular zone of adult mice, and the expression of neuronal-NOS or epidermal-NOS was observed to have increased during the differentiation of cells[12]. Nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) is a histochemical marker specific for NOS in the central nervous system (CNS)[13]. Neurons containing

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NADPH-d have been reported to be relatively resistant to various toxic insults and neurodegenerative diseases[14]. Our previous study demonstrated that NOS expression in the cerebral cortex of rats was decreased by alcohol treatment[15]. It was also reported that aqueous extracts of *Artemisia capillaries* possesses preventive effect against ethanol-induced apoptosis in neuroblastoma cell line[16,17], and *Puerariae flos* was also shown to have anti-apoptotic effect against alcohol[18]. In the present study, the effect of *Puerariae radix* on cell proliferation and NOS expression in the dentate gyrus of acutely alcohol-intoxicated rats were investigated via 5-bromo-2'deoxyuridine (BrdU) imunohistochemmistry and NADPH-d histochemistry, respectively.

Materials and Methods

1. Animals and treatment

Male Sprague-Dawley rats weighing 150 ± 10 g (5 weeks in age) were used in the present study. The experimental procedures were conducted in accordance with the animal care guidelines of the NIH and the Korean Academy of Medical Sciences. Each animal was housed at a controlled temperature (20 ± 2℃) 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*.
To obtain aqueous extracts of *Puerariae radix*, 200 g of *Puerariae radix* was added to distilled water, heat-extracted, concentrated with a rotary evaporator and lyophilized. The resulting powder, weighing 35 g (a collection rate of 17.5 %) was diluted with saline solution. Animals were divided into four groups: the control group, the *Puerariae radix*-treated group, the alcohol-treated group, and the alcohol- and *Puerariae radix*-treated group (n = 5 in each group). Rats of the control group were injected intraperitoneally with BrdU (50 mg/kg; Sigma, St. Louis, MO, USA) for 3 consecutive days, while animals of the *Puerariae radix*-treated group were injected with an equivalent dose of BrdU and 30 mg/kg of *Puerariae 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 the same duration of time. Animals of the *Puerariae radix*- and alcohol-treated group were injected with BrdU, alcohol, and *Puerariae radix* extracts in doses used on animals of other groups for 3 days. Blood was collected from animals *via* cardiac puncture 2 h after the last alcohol injection, and serum alcohol concentration was measured using a Sigma Diagnostics Kit (Sigma Chemical Co., St. Louis, MO, USA).

2. Tissue preparation

For the sacrificial process, animals were first fully anesthetized with Zoletil 50® (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).

3. BrdU immunohostochemistry

For detection of newly generated cells in the dentate gyrus, the associated BrdU incorporation was visualized *via* a previously described immunohistochemical method[2,3]. First, ten 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). The sections were incubated for another 1 h with VECTASTAIN® Elite ABC Kit (1:100; Vector Laboratories, Burlingame, CA, USA). For immunostaining, 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, counter-staining was performed on the same sections using a mouse anti-neuronal nuclei antibody (1;300; Chemicon International, Temecula, CA, USA). The sections were then washed three times with PBS, incubated for 1 h with a biotinylated mouse secondary antibody, and processed with VECTASTAIN® ABC Kit. For immunostaining, the sections were incubated in 0.02% DAB (40 mg/ml) and 0.03% hydrogen peroxide in 50 mM Tris-HCl for 5 min, and then washed with PBS and mounted onto gelatin-coated slides.

4. NADPH-d histochemistry

Sections were stained for NADPH-d activity according to a previously described protocol[14,15]. 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 Permount .

5. 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 data was expressed as number of cells per mm^2 of cross-sectional area of the granular layer of the dentate gyrus[3,14]. Statistical differences were determined using ANOVA followed by Scheffe's post-hoc analyses, and results were expressed as mean ± S.E.M. Differences were considered significant for $P < 0.05$.

Results

1. Serum alcohol concentration in each group

The serum alcohol concentration was 82.04 ± 3.78 mg/dl in the alcohol treated groups and 0 or negligible in the control group and *Puerariae radix* treated-group.

2. Number of BrdU-positive cells in dentate gyrus of each group

BrdU-positive cells in each group are presented in Fig. 1.

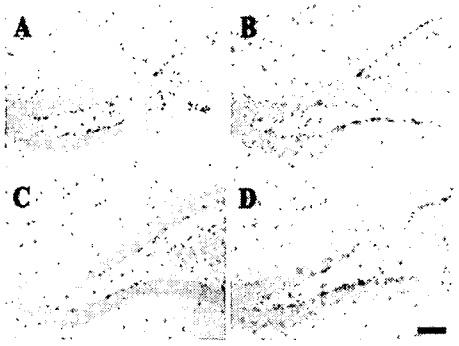

Fig. 1. Photomicrograph of 5-bromo-2'-deoxyuridine (BrdU)-positive cells. Sections were stained for BrdU (black) and neuronal nuclei (NeuN) (brown). Scale bar represents 100 ㎛. A: control group, B: *Puerariae radix*-treated group, C: alcohol-treated group, D: alcohol- and *Puerariae radix*-treated group.

The number of BrdU-positive cells in the dentate gyrus was about 292.80 ± 10.88/mm^2 in the control group, 310.80 ± 11.36/mm^2 in the *Puerariae radix*-treated group, 133.43 ± 12.77/mm^2 in the alcohol-treated group, and 301.33 ± 8.74/mm^2 in the alcohol- and *Puerariae radix*-treated group (Fig. 2). Number of BrdU-positive cells in the dentate gyrus was suppressed significantly by alcohol administration. *Puerariae radix* increased new cell formation in the dentate gyrus in alcohol-intoxicated rats, while it exerted no specific effect on cell proliferation under normal conditions.

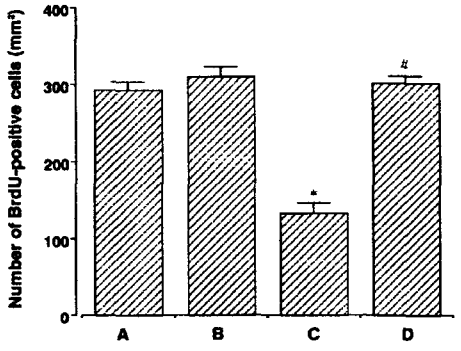

Fig. 2. Mean number of 5-bromo-2'-deoxyuridine (BrdU)-positive cells in the subgranular layer of the dentate gyrus in each group. A: control group, B: *Puerariae radix*-treated group, C: alcohol-treated group, D: alcohol- and *Puerariae radix*-treated group. Values are represented as mean ± S.E.M. * represents $P < 0.05$ compared to the control group. # represents $P < 0.05$ compared to the alcohol-treated group.

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

NADPH-d-positive cells in each group are presented in Fig. 3.

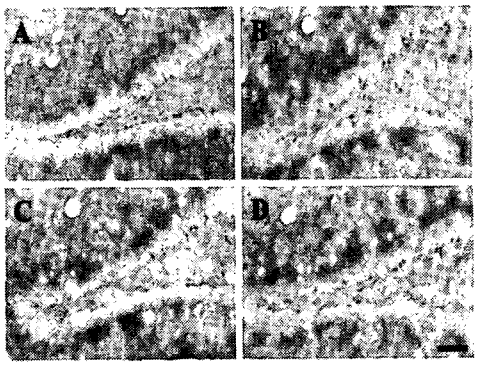

Fig. 3. Photomicrograph of nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d)-positive cells. Sections were stained for nitric oxide synthase (blue). Scale bar represents 100 ㎛. A: control group, B: *Puerariae radix*-treated group, C: alcohol-treated group, D: alcohol- and *Puerariae radix*-treated group.

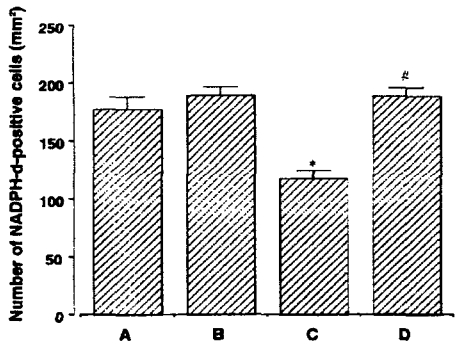

Fig. 4. Mean number of nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d)-positive cells in the dentate gyrus in each group. A: control group, B: *Puerariae radix*-treated group, C: alcohol-treated group, D: alcohol- and *Puerariae radix*-treated group. Values are represented as mean ± S.E.M. * represents $P < 0.05$ compared to the control group. # represents $P < 0.05$ compared to the alcohol-treated group.

The number of NADPH-d-positive cells in the dentate gyrus was about 177.16 ± 10.88/mm^2 in the control group,

189.44 ± 6.63/mm^2 in the *Puerariae radix*-treated group, 117.60 ± 6.70/mm^2 in the alcohol-treated group, and 189.00 ± 6.79/mm^2 in the alcohol- and *Puerariae radix*-treated group (Fig. 4). Number of NADPH-d-positive cells in the dentate gyrus was suppressed significantly by alcohol administration. *Puerariae radix* increased NADPH-d expression in the dentate gyrus in alcohol-intoxicated rats, while it exerted no specific effect on NOS expression under normal conditions.

Discussion

Puerariae radix, one of the most commonly encountered Oriental herbs in the treatment of alcohol-related problems, is known to inhibit mitochondrial aldehyde dehydrogenase and to suppress ethanol intake in Syrian golden hamsters[1]. In this study, the effect of *Puerariae radix* on cell proliferation and nitric oxide synthase (NOS) expression in the dentate gyrus of alcohol-intoxicated Sprague-Dawley rats were investigated via BrdU immunohistochemistry and NADPH-d histochemistry.

In the present study, it was demonstrated that aqueous extracts of *Puerariae radix* increases BrdU-positive and NADPH-d-positive cells only slightly under normal conditions, with statistical insignificance, while these numbers were decreased significantly by alcohol administration. The alcohol-induced inhibition of new cell formation in the dentate gyrus seen in the present results points at a probable reduction in newly formed granule neurons. It may be suggested that the decrease in learning capability and memory function induced by alcohol[8] is related to the inhibitory action of alcohol on cell proliferation[15]. It has been suggested that NO plays a critical role in the formation of new neurons after birth and that it regulates neurogenesis in the adult CNS[11]. Increasing evidence shows that alcohol inhibits NO production in vivo[10,19]. In our previous study, it was shown that decreased NOS expression in the cerebral cortex by alcohol administration is one of the major mechanisms of alcohol-induced memory and learning impairments[15]. It has also been reported that aqueous extracts of *Artemisia capillaries* exerts anti-apoptotic effect against alcohol-induced apoptosis in neuroblastoma cells and the inhibitory action of *Artemisia capillaries* on *bax* and *caspase-3* mRNA expression was suggested as one of the important anti-apoptotic mechanisms of *Artemisia capillaries* on alcohol-induced apoptosis[16,17]. In addition, *Puerariae flos* was shown to exert the preventive effect against alcohol-induced apoptosis *via* inhibiting *caspase-3* mRNA expression in neuroblastoma cells[18].

In the present results, *Puerariae radix* treatment was shown to increase the numbers of both BrdU-positive and NADPH-d-positive cells in the dentate gyrus of alcohol-intoxicated rats. In the present study, it was demonstrated that aqueous extracts of *Puerariae radix* exert protective effect against alcohol-induced suppression in new cell formation, and it is possible that NO, which might play an important role in the regulation of cell proliferation, is a major target of the toxic effects of alcohol.

Acknowledgments

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