Effects of \textit{Puerariae radix} on Cell Proliferation and Nitric Oxide Synthase Expression in Dentate Gyrus of Alcohol-Intoxicated Sprague-Dawley Rats

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ABSTRACT—Traditionally, \textit{Puerariae radix} had been used for the treatment of alcohol-related problems. In this study, effects of \textit{Puerariae radix} on cell proliferation and nitric oxide synthase expression in the dentate gyrus of alcohol-intoxicated Sprague-Dawley rats were investigated via 5-bromo-2'-deoxyuridine (BrdU) immunohistochemistry and nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) histochemistry. Alcohol administration was shown to inhibit the numbers of both BrdU-positive and NADPH-d-positive cells, while \textit{Puerariae radix} treatment was shown to increase those numbers. It is possible that nitric oxide, which might play an important role in the regulation of cell proliferation, is a major target of the toxic effects of alcohol.

Keywords: \textit{Puerariae radix}, Alcohol, Immunohistochemistry

Alcohol consumption is known to cause substantial neuronal loss in several regions of the brain (1 – 3). It has been reported that alcohol induces death in a variety of cells including astroglia (1) and neuroblastoma cells (2) in vitro and that it triggers apoptotic neurodegeneration in the developing rat brain in vivo (3). In addition, alcohol intake during the developmental stage has been correlated with deficits in learning and memory (4).

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 (5, 6). 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 (5 – 7). However, no study on the effect of alcohol on cell proliferation has been published to date.

Nitric oxide (NO), synthesized from \textit{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 (8). Nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) is a histochemical marker specific for NOS in the central nervous system (CNS) (9). Neurons containing NADPH-d have been reported to be relatively resistant to various toxic insults and neurodegenerative diseases (10). 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 (11). Moreno-López et al. (12) 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 (13).

Traditionally, \textit{Puerariae radix} has been used as an antipyretic, anti-diarrheic, diaphoretic and antiemetic agent. In Oriental medicine, medications based on \textit{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 (14). In the present study, the effects of \textit{Puerariae radix} on cell proliferation and NOS expression in the dentate gyrus of rats in the process of growing up
which had been acutely intoxicated with alcohol were investigated via 5-bromo-2’-deoxyuridine (BrdU) immunohistochemistry and NADPH-d histochemistry, respectively.

Male Sprague-Dawley rats weighing 90 ± 10 g were used in the present study. The experimental procedures were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society 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 0.3 mg/kg *Puerariae radix*-treated group, the alcohol- and 3 mg/kg *Puerariae radix*-treated group, the alcohol- and 30 mg/kg *Puerariae radix*-treated group, and the alcohol- and 300 mg/kg *Puerariae radix*-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) for 3 consecutive days, whereas animals of the alcohol-treated group were injected with 50 mg/kg of BrdU and 2 g/kg of alcohol for the same duration of time. Animals of the alcohol- and *Puerariae radix*-treated groups were injected with BrdU and alcohol in doses used on other groups with *Puerariae radix* extracts over the respective dose of the groups for 3 days.

To obtain 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.

Blood was collected from animals via cardiac puncture 2 h after the last injection, and serum alcohol concentration was measured using a Sigma Diagnostics® Kit (Sigma Chemical Co.). 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 0.1 M 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-μm thickness were made with a freezing microtome (Leica, Nußloch, Germany).

For detection of newly generated cells in the dentate gyrus, the associated BrdU incorporation was visualized via a previously described immunohistochemical method (5, 6). 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 they were pretreated in 50% formamide-2X standard saline citrate (SSC) at 65°C for 2 h, denatured in 2 N HCl at 37°C for 30 min, and rinsed twice in 0.1 M sodium borate (pH 8.5). Afterwards, the sections were incubated overnight at 4°C 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).

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.

Sections were stained for NADPH-d activity according to a previously described protocol (10). In brief, free-floating sections were incubated at 37°C for 60 min in 0.1 M 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 gelatin-coated slides. The slides were air dried overnight at room temperature, and coverslips were mounted using Permount®.

The area of the dentate gyrus region was measured hemilaterally in each of the selected sections using an image analyzer (Multiscan; American Applied Biotechnology, 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 (6, 10). Statistical differences were determined using Student’s *t*-test, and results were expressed as mean ± S.E.M. Differences were considered significant for *P*<0.05.

The serum alcohol concentration was 70.94 ± 3.62 mg/dl in the alcohol-treated group and 0 or negligible in the control group. The number of BrdU-positive cells in the control group was 262.00 ± 19.30/mm², and this figure was 121.78 ± 13.44/mm² for the alcohol-treated group. The number of BrdU-positive cells was 107.64 ± 8.69/mm².
in the group treated with alcohol and 0.3 mg/kg *Puerariae radix*, which is not indicative of any noticeable change, but this figure rose to 224.40 ± 17.22/mm² for the group treated with alcohol and 3 mg/kg *Puerariae radix* and to
was 153.20 ± 4.72/mm² for the alcohol-treated group. For the group treated with alcohol and 0.3 mg/kg *Puerariae radix*, the number of NOS-positive cells was 111.60 ± 8.77/mm², and this figure was 167.50 ± 7.20/mm² and 185.50 ± 8.62/mm² for the group treated with alcohol and 3 mg/kg *Puerariae radix* and that treated with alcohol and 30 mg/kg *Puerariae radix*, respectively. This trend was reversed in the group treated with alcohol and 300 mg/kg *Puerariae radix*, with the number of NOS-positive cells at 138.00 ± 11.83/mm² (Fig. 2). These results indicate that *Puerariae radix* possesses the protective effect against alcohol-induced inhibition on NOS expression.

In the present study, it was demonstrated that alcohol administration decreases BrdU-positive and NADPH-d-positive cells significantly. 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 is related to the inhibitory action of alcohol on cell proliferation. 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 (12). Increasing evidence shows that alcohol inhibits NO production in vivo (15).

*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 (14). 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 decrease 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.

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**REFERENCES**