Effect of \textit{Puerariae radix} on c-Fos Expression in Hippocampus of Alcohol-Intoxicated Juvenile Rats

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Received March 8, 2002; accepted September 17, 2002

Alcohol consumption is known to cause substantial neuronal loss in several regions of the brain. In Oriental medicine, medications based on \textit{Puerariae radix} have been known to be of efficacy in the treatment of alcohol-related problems. In the present study, the effect of the aqueous extract of \textit{Puerariae radix} on the expression of c-Fos, an immediate early gene whose expression is sometimes used as a marker for stimulus-induced changes in the metabolic activity of neurons, was investigated via immunohistochemistry. In the first part of the experiment, Sprague-Dawley rats were divided into six groups: the control group, the alcohol-treated group, the alcohol- and 0.3 mg/kg \textit{Puerariae radix}-treated group, the alcohol- and 3 mg/kg \textit{Puerariae radix}-treated group, the alcohol- and 30 mg/kg \textit{Puerariae radix}-treated group, and the alcohol- and 300 mg/kg \textit{Puerariae radix}-treated group. In the second part of the experiment, animals were divided into four groups: the control group, the 30 mg/kg \textit{Puerariae radix}-treated group, the alcohol-treated group, and the alcohol- and 30 mg/kg \textit{Puerariae radix}-treated group. From the results, it was demonstrated that alcohol administration significantly decreases the number of Fos-positive cells in the various regions of the hippocampus, and \textit{Puerariae radix} treatment inhibits the alcohol-induced suppression of the expression of Fos in the hippocampus in a dose-dependent manner. \textit{Puerariae radix} exerted no significant effect on Fos expression in the hippocampus of normal rats. The results presented in this study suggest that \textit{Puerariae radix} may alleviate alcohol-induced disruption of hippocampal functions.

\textbf{Key words} \textit{Puerariae radix}; c-Fos; alcohol; hippocampus; immunohistochemistry

Traditionally, \textit{Puerariae radix} has been used as an antipyretic, antidiarrheic, 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 antiintoxication and antidrinking agent, and in the treatment of various liver diseases caused by alcohol abuse.\(^1\)

Alcohol consumption is known to cause substantial neuronal loss in several regions of the brain,\(^2\)–\(^4\) and to exert various effects on the central nervous system (CNS).\(^5\) It has been reported that alcohol induces death in a variety of cells including astroglia\(^6\) and neuroblastoma cells \textit{in vitro}\(^7\) and that it triggers apoptotic neurodegeneration in the developing rat brain \textit{in vivo}.\(^8\) In addition, alcohol intake during the developmental stage has been associated with deficits in learning and memory,\(^9,10\) and alcohol abuse has been shown to induce behavioral disorders during adolescence.\(^11\)

The hippocampal formation is a brain region critically involved in learning and memory formation. It has been documented that acute alcohol intoxication disrupts performance in short-term memory tasks in rodents and humans.\(^9,10\) Recently, it was reported that alcohol intoxication produces regional selective changes in the expression of inducible transcription factors and metabolism in the brain.\(^11,12\)

c-Fos is an immediate early gene whose expression is sometimes used as a marker for stimulation-induced changes in the metabolic activity of neurons.\(^13\) Melia \textit{et al.}\(^14\) have reported that alcohol treatment suppresses the expression of c-fos in the dentate gyrus (DG) of the hippocampus. In the present study, the effect of the aqueous extract of \textit{Puerariae radix} on c-Fos expression in the hippocampus of acutely alcohol-intoxicated juvenile rats was investigated via immunohistochemistry.

\textbf{MATERIALS AND METHODS}

\textbf{Animals and Treatment} Male Sprague-Dawley rats weighing 90±10 g (30 d postnatal) were used for the experiment. The experimental procedures were performed 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 °C) and maintained under ligh–dark cycles, each consisting of 12 h of light and 12 h of darkness, with food and water made available \textit{ad libitum}. To obtain the extract of \textit{Puerariae radix}, 200 g of \textit{Puerariae radix} was added to distilled water, heat extracted, concentrated with a rotary evaporator, and lyophilized. The resulting powder weighing 35 g (a yield of 17.5%) was diluted with saline.

In the first part of the experiment, animals were divided into six groups: the control group, the alcohol-treated group, the alcohol- and 0.3 mg/kg \textit{Puerariae radix}-treated group, the alcohol- and 3 mg/kg \textit{Puerariae radix}-treated group, the alcohol- and 30 mg/kg \textit{Puerariae radix}-treated group, and the alcohol- and 300 mg/kg \textit{Puerariae radix}-treated group (\(n=5\) for each group).

In the second part of the experiment, animals were divided into four groups: the control group, the 30 mg/kg \textit{Puerariae radix}-treated group, the alcohol-treated group, and the alcohol- and 30 mg/kg \textit{Puerariae radix}-treated group (\(n=5\) for each group).

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each group).

Rats in the control group were injected intraperitoneally with saline once a day for 3 consecutive days, while animals in the alcohol-treated group were injected once a day with 2 g/kg of alcohol for the same duration of time. Animals in the alcohol- and *Puerariae radix*-treated groups were injected with 2 g/kg of alcohol and the appropriate amount of *Puerariae radix* extract once a day for 3 d.

**Blood Alcohol Concentration Measurement** For analysis of serum alcohol concentration, blood was collected from animals via cardiac puncture 2 h after the last alcohol injection, and the blood alcohol concentration was measured using a Sigma Diagnostics kit (Sigma Chemical Co., St. Louis, MO, U.S.A.) according to the manufacturer’s protocol.

**Tissue Preparation** Animals were first fully anesthetized with Zoletil 50® (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 40 μm thick were made with a freezing microtome (Leica, Nussloch, Germany).

**Fos Immunohistochemistry** Fos immunostaining was performed according to a protocol described by He et al. The c-Fos antibody used in this study was provided as a rabbit affinity purified polyclonal antibody raised against a peptide mapping at the amino terminus of c-Fos p62 of human origin (identical to the corresponding mouse sequence).

Eight sections on average were selected from each brain region spanning from Bregma −3.30 to −4.16 mm. Free-floating tissue sections were incubated overnight with rabbit anti-Fos antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) at a dilution of 1:1000, and the sections were then incubated for 1 h with biotinylated anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, U.S.A.). The sections were subsequently incubated with avidin–biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, U.S.A.) for 1 h at room temperature. Immunoreactivity was visualized by incubating the sections in a solution consisting of 0.05% 3,3-diaminobenzidine and 0.01% H2O2 in 50 mM Tris–buffer (pH 7.6) for approximately 3 min. As the negative control, brain sections from the experiment were likewise processed using normal goat serum as the primary antibody; no c-Fos-like immunoreactivity was observed.

**Data Analyses** To quantify the number of Fos-positive cells in each area of the hippocampus, cell counting was performed under a light microscope (Olympus, Tokyo, Japan). The number of Fos-positive cells inside the pyramidal cell layer was counted hemilaterally in each of the selected hippocampal regions.

**Statistical Analysis** Statistical significance of differences were determined by one-way analysis of variance (ANOVA) followed by Scheffe’s post-hoc analysis, and results were expressed as mean±standard error mean (S.E.M.) of Fos-positive cells. Differences were considered significant for *p*<0.05.

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**RESULTS**

**Blood Alcohol Concentration** The serum alcohol concentration was about 71.62±4.51 mg/dl in the alcohol-treated groups and 0 or negligible in the control group.

**Fos-Positive Cells in the CA1 Region of the Hippocampus** From the results of the first part of the experiment, the number of Fos-positive cells in the CA1 region of the hippocampus in the control group was 163.50±8.86 per section, and this figure was 82.00±7.46 for the alcohol-treated group. The number of Fos-positive cells in the group treated with alcohol and 0.3 mg/kg *Puerariae radix* was 75.90±4.16 per section, which is not indicative of any significant change, but this figure rose to 111.10±6.19 for the group treated with alcohol and 3 mg/kg *Puerariae radix,* to 119.80±3.48 for the group treated with alcohol and 30 mg/kg *Puerariae radix,* and to 123.57±2.72 for the group treated with alcohol and 300 mg/kg *Puerariae radix* (Fig. 1).

From the results of the second part of the experiment, the number of Fos-positive cells in the CA1 region of the hippocampus in the control group was 140.20±9.80 per section, 158.20±10.24 per section in the 30 mg/kg *Puerariae radix*-treated group, which is not indicative of any significant change, and 74.90±8.41 per section for the alcohol-treated
group. But this figure rose to 152.30±12.33 per section for the alcohol- and 30 mg/kg *Puerariae radix*-treated group (Fig. 2).

**Fos-Positive Neurons in the CA2 and CA3 Regions of the Hippocampus** From the results of the first part of the experiment, the number of Fos-positive cells in the CA2 and CA3 regions of the hippocampus in the control group was 66.70±2.62 per section, and this figure was 43.90±2.83 for the alcohol-treated group. The number of Fos-positive cells in the group treated with alcohol- and 0.3 mg/kg *Puerariae radix* was 39.30±1.68 per section, which is again not indicative of any significant change, but this figure rose to 82.40±4.29 for the group treated with alcohol and 3 mg/kg *Puerariae radix*, to 87.00±2.89 for the group treated with alcohol and 30 mg/kg *Puerariae radix*, and to 80.80±3.44 for the group treated with alcohol and 300 mg/kg *Puerariae radix* (Fig. 3).

From the results of the second part of the experiment, the number of Fos-positive cells in the CA2 and CA3 regions of the hippocampus in the control group was 71.60±4.31 per section, 74.40±5.68 per section in the 30 mg/kg *Puerariae radix*-treated group, which is not indicative of any significant change, and 41.10±3.20 per section for the alcohol-treated group. But this figure rose to 80.90±8.23 per section for the alcohol- and 30 mg/kg *Puerariae radix*-treated group (Fig. 2).

**Fos-Positive Neurons in the Dentate Gyrus of the Hippocampus** From the results of the first part of the experiment, the number of Fos-positive cells in the dentate gyrus of the hippocampus in the control group was 81.00±3.37 per section, and this figure was 33.20±2.87 for the alcohol-treated group. The number of Fos-positive cells in the group treated with alcohol- and 0.3 mg/kg *Puerariae radix* was 45.80±3.81 per section, and this figure rose further, to 82.80±3.35 for the group treated with alcohol and 3 mg/kg *Puerariae radix*, to 83.10±1.75 for the group treated with alcohol and 30 mg/kg *Puerariae radix*, and to 79.43±4.59 for the group treated with alcohol and 300 mg/ml kg *Puerariae radix* (Fig. 4).

From the results of the second part of the experiment, the number of Fos-positive cells in the dentate gyrus region of the hippocampus in the control group was 79.40±3.84 per section, 81.40±7.72 per section in the 30 mg/kg *Puerariae radix*-treated group, which is not indicative of any significant change, and 33.90±3.41 per section for the alcohol-treated group. But this figure rose to 83.60±7.92 per section for the alcohol- and 30 mg/kg *Puerariae radix*-treated group (Fig. 2).

**DISCUSSION**

In the present study, it was demonstrated that alcohol administration significantly decreases the number of Fos-positive cells in the various hippocampal regions. It has been reported that alcohol induces death in astroglia and neuroblastoma cells and that ethanol consumption during CNS development leads to substantial neuronal loss in multiple brain regions.1,2,3,4 Electrophysiological studies suggest that alcohol disrupts learning and memory formation by compromising hippocampal functions.10 At physiologically relevant concentrations, ethanol was shown to diminish the amplitude of stimulation-evoked spikes in hippocampal slices, suggesting a suppressive effect of alcohol on cell activity.10 It has thus been sug-
the most commonly utilized 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.\(^1\) \textit{Puerariae radix} is comprised of puerarin, daidzin, daidzein, genistein, and biochanin \(A\); of these, daidzin and daidzein, the major active constituents of \textit{Puerariae radix} extracts, are known to suppress ethanol intake.\(^{18,20}\) In a previous study, the inhibitory effect of \textit{Puerariae flos} on alcohol-induced apoptosis, which appears to act by inhibiting \textit{caspase}-3 mRNA expression, in neuroblastoma cells was reported.\(^{21}\)

In the present study, \textit{Puerariae radix} treatment increased the number of Fos-positive cells in the hippocampus of alcohol-intoxicated rats in a dose-dependent manner, while \textit{Puerariae radix} exerted no significant effect on Fos expression in the hippocampus of normal rats. The results presented in this study suggest that \textit{Puerariae radix} may alleviate alcohol-induced disruption of hippocampal functions.

**Acknowledgements** This study was supported by a grant from the Pain and Neuroscience Research program of the Vision 2000 Project of Kyung Hee University.

**REFERENCES**