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五加皮 藥針이 알코올 中毒 흰쥐의 齒狀回에서 神經細胞生成 및 NOS 發顯에 미치는 影響

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Effects of Acanthopanax senticosus herb-acupuncture on cell proliferation and nitric oxide synthase expression in dentate gyrus of alcohol-intoxicated rats

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Abstract

목적 : 오가피 약침이 알코올에 의해서 중독된 Sprague-Dawley(S-D)계 흰쥐의 해마 치상회에서 새로운 신경세포 생성 및 NOS발현에 미치는 영향을 조사하였다.

방법 : S-D계 흰쥐에 알코올을(2g/kg) 3일간 연속으로 투여한후, 5일간 인체의 중완혈에 상응하는 부위에 오가피 약침(30mg/kg) 치료를 시행하였다. 치료효과를 관찰하기 위해서 BrdU-면역조직화학 염색법 및 NADPH-d-조직화학염색법을 이용하였다.

결과 : 알코올 처치군에서는 BrdU-양성세포수 및 NADPH-양성세포수가 모두 정상군에 비해서 감소한 반면에 알코올 처치후 오가피 약침으로 치료한 군에서는 BrdU-양성세포수 및 NADPH-양성세포수 모두 알코올 처치군에 비해서 증가하였다.

결론 : 오가피 약침치료가 알코올에 의해서 중독된 S-D계 흰쥐 해마 치상회에서 새로운 신경세포의 생성을 증가시키는 것을 확인하였으며, 그 기전으로 산화질소가 관여할 것으로 사려된다.

Key words : *Acanthopanax senticosus*; Alcohol; Immunohistochemistry.

Ⅰ. INTRODUCTION

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].

Loss of neurons is thought to be irreversible in the adult human brain, because dying neurons cannot be replaced.

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This inability to generate replacement cells is thought to be an important cause of neurological disease and impairment. In most brain regions, the generation of neurons is generally confined to a discrete developmental period. Exceptions are found in the dentate gyrus and the subventricular zone of several species that have been shown to generate new neurons well into the postnatal and adult period[5-10]. Granule neurons are generated throughout life from a population of continuously dividing progenitor cells residing in the subgranular zone of the dentate gyrus in the rodent brain[9]. 'Newborn' neurons generated from these progenitor cells migrate into the granule cell layer, differentiate, extend axons and express neuronal marker proteins[11-14]. 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[15,16]. 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[15-17]. However, no study on the effect of alcohol on cell proliferation has been published to date.

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[18]. Nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH -d) is a histochemical marker specific for NOS in the central nervous system (CNS)[19]. Neurons containing NADPH-d have been reported to be relatively resistant to various toxic insults and neurodegenerative diseases[20]. 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[21]. Moreno- Lopez et al.[22] 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[23]. Traditionally, *Acanthopanax senticosus* (AS), a member of the araliaceae family, is a herb which has been used traditionally for improvement from ischemic injury, rheumatism, weakened physical status, and for its hypoglycemic action[24-26].

In the present study, the effects of *AS* 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.

Ⅱ. MATERIAL AND METHODS

1. Animals

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 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*. Animals were divided into four groups: the control group, the *AS*-treated group, the alcohol-treated group, and the alcohol- and *AS*-treated group (n = 6 for 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 *AS*-treated group were injected with an equivalent dose of BrdU and 30 mg/kg of *AS* 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 *AS*- and alcohol-treated group were injected with BrdU, alcohol, and *AS* extracts in doses used on animals of other groups for 3 days.

2. Reagents

To obtain extracts of *AS*, 200 g of *AS* 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.

3. Tissue preparation

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 (St. Louis, MO, USA). For the sacrificial process, animals were first fully anesthetized with Zoletil (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 ㎛ thickness were made with a freezing microtome (Leica, Nuloch, Germany).

4. BrdU immunohistochemisty

For detection of newly generated cells in the dentate gyrus, the associated BrdU incorporation was visualized *via* a previously described immunohistochemical method (15,16). 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-2X standard saline citrate (SSC)

at 65 ℃ for 2 h, denaturated in 2 N HCl at 37 ℃ for 30 min, and rinsed twice in 0.1 M 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.

5. NADPH-d immunohistochemistry

Sections were stained for NADPH-d activity according to a previously described protocol[20]. In brief, free-floating sections were incubated at 37 ℃ 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 gelatine-coated slides. The slides were air dried overnight at room temperature, and coverslips were mounted using Permount.

6. Statistical 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 total numbers of BrdU-positive and NADPH-d-positive cells were obtained, and the results were expressed as number of cells per mm^2 of cross-sectional area of the granular layer of the dentate gyrus[16,20]. Statistical differences were determined using Student's *t*-test, and results were expressed as mean ± S.E.M. Differences were considered significant for $P < 0.05$.

Ⅲ. RESULTS

1. The number of BrdU-positive cells in the dentate gyrus

The number of BrdU-positive cells in the dentate gyrus was about 292.80± 10.88/mm^2 in the control group, 133.43± 12.77/mm^2 in the alcohol-treated group, 310.80±11.36/mm^2 in the *AS*-treated group,

Table I. Effect of *Acanthopanax senticosus* on cell proliferation in dentate gyrus of alcohol-intoxicated rats

	No. of animals	BrdU-positive cells (No./mm^2)	Duncan Grouping
Control	6	292.80 ± 10.88[1)]	A[2)]
Alcohol	6	133.43 ± 12.77	B
Sample A	6	310.80 ± 11.36	A
Sample B	6	301.33 ± 8.74	A

1) Mean ± Standard error of 6 rats
2) The same letter are not significantly different at the α=0.05 level by Duncan test.
Control : Untreated group.
Alcohol : Alcohol treated group
Sample A : *Acanthopanax senticosus (AS)*-treated group
Sample B : Alcohol- and *AS*-treated group

and 301.33±8.74/mm^2 in the alcohol- and *AS*-treated group (Table I).

2. The number of NADPH-d-positive cells in the dentate gyrus

The number of NADPH-d-positive cells in the dentate gyrus was about 177.16±10.88/mm^2 in the control group, 117.60±6.70/mm^2 in the alcohol-treated group, 189.44±6.63/mm^2 in the *AS*-treated group, and 189.00±6.79/mm^2 in the alcohol- and *AS*-treated group (Table II).

Table Ⅱ. Effect of *Acanthopanax senticosus* on NADPH-d-positive expression in dentate gyrus of alcohol-intoxicated rats

	No. of animals	BrdU-positive cells (No./mm^2)	Duncan Grouping
Control	6	177.16±10.88[1)]	A[2)]
Alcohol	6	117.60±6.70	B
Sample A	6	189.44±6.63	A
Sample B	6	189.00±6.79	A

1) Mean ± Standard error of 6 rats
2) The same letter are not significantly different at the α=0.05 level by Duncan test.
Control : Untreated group.
Alcohol : Alcohol-treated group
Sample A : *Acanthopanax senticosus (AS)*-treated group
Sample B : Alcohol- and *AS*-treated group

Ⅳ. DISCUSSION

Ethanol consumption leads to neuronal death and causes devastating pathologies within the central nervous system[28)]. In spite of over 20 years of experimental and clinical efforts the mechanism by which ethanol causes neuronal death is not entirely clear. Some of these pathologies has been linked to thiamine deficiency which often accompanies chronic ethanol consumption[29)]. However, recent electrophysiological, neurochemical and behavioral studies provided evidence that several ligand-gated ion channels are especially sensitive to ethanol. These ethanol-sensitive ion channels include NMDA and $GABA_A$ receptors[30)]. Ethanol, when administrated acutely, inhibits NMDA-induced ion currents *in vivo*[31)] and reduces NMDA-evoked neurotoxicity[32)]. In contrast, chronic exposure to ethanol enhances NMDA receptor function[33)] and therefore potentiates NMDA-induced neurotoxicity[34)]. Consequently, circadian variations in ethanol concentration may result in ethanol mini-withdrawals with subsequent NMDA-receptor overstimu-

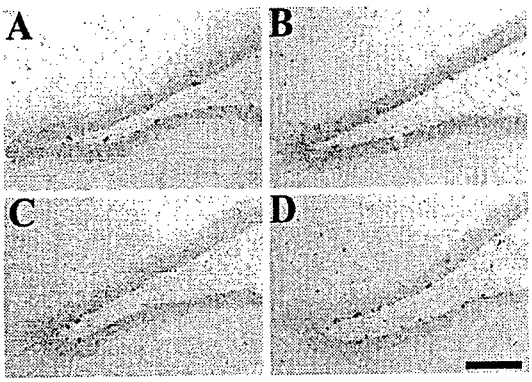

Fig. 1. Effect of Acanthopanax senticosus (AS) on cell proliferation. A; control group, B; AS-treated group, C; alcohol-treated group, D; alcohol-and AS-treated group. Scale bar represents 100 ㎛.

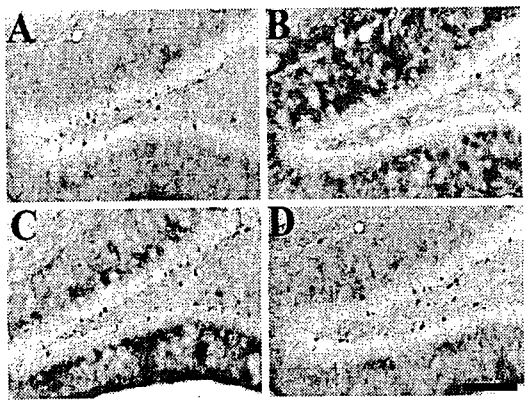

Fig. 2. Effect of *Acanthopanax senticosus* (AS) on nitric oxide synthase expression. A; control group, B; AS-treated group, C; alcohol-treated group, D; alcohol-and AS-treated group. Scale bar represents 100 ㎛.

lation[35] resulting in neuronal death and subsequent cognitive impairments.

One of the putative mechanisms for central nervous system to regenerate is a production of new neurons. In almost all regions of the mammalian brain neurogenesis occurs only during the prenatal period. However, in the dentate gyrus of several species, including mice and humans, the granyule cells are generated postnatal[36,37]. The newborn neurons are capable of extending their axons and reach the CA3 area of the hippocampus, therefore they seem to be functionally active[38].

Nitric oxide (NO) is a free radical molecule with signaling functions in the central nervous system. In the brain parenchyma, NO is synthesized by widely distributed specific neurons that express the neuronal isoform of NO synthase (NOS I)[39]. The transient expression of NOS I in different neural structures during development suggests that NO plays a role in embryonic neurogenesis. The participation of NO in the formation of the nervous system may be related to its involvement in neuronal programmed cell death, in the organization of axonal projection patterns, or in the control of cell proliferation[22]. In neuronal cell lines, NOS I and II isoforms are induced by differentiating agents and, in some cases, the NO antiproliferative action was shown to be a prerequisite for differentiation. Concerning neurogenesis in the SVZ, NO is an antimitotic agent in primary cultures of proliferating cells isolated from this region[40].

In the present study, it was demonstrated that *AS* increases BrdU- positive and NADPH-d-positive cells 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 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[22]. Increasing evidence shows that alcohol inhibits NO production *in vivo*[25]. *AS*, 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[24]. In the present results, *AS* treatment was shown to increase the numbers of both BrdU-positive and NADPH-d-positive cells in the dentate gyrus of alcohol-intoxicated rats.

V. CONCLUSION

In the present study, it was demonstrated that aqueous extracts of *AS* 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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