Protective Effect of Ginseng Saponins against Impaired Brain Growth in Neonatal Rats Exposed to Ethanol

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This study was performed to determine the active constituents of the root of Panax ginseng C. A. Mayer in the amelioration of ethanol-induced impediment of brain growth in the neonatal stage. To establish an animal model of the brain growth impediment caused by ethanol, ethanol (6 g/kg s.c.) was administered to rat pups on postnatal day 6, which corresponded to the third trimester of pregnancy for humans. Brain weight, especially cerebellar weight, was significantly reduced in the ethanol-exposed pups. In contrast, neither separation from dams nor pentobarbital treatment affected brain weight. A saponin fraction of ginseng extract prevented this ethanol-induced reduction of brain weight. Some ginseng saponins including ginsenosides Rg₁, Rb₂, Rd, Rf and Re effed stimulatio a potent recovery of cerebellum growth in this animal model.

Keywords: ethanol; cerebellum; neonatal stage; Panax ginseng; ginseng saponin

Ethanol use during pregnancy has been widely described as having a significant negative effect on normal fetal development. The most devastating consequence is the suboptimal performance of the central nervous system (CNS) in infants, which has been labeled fetal alcohol syndrome (FAS). The CNS is vulnerable to ethanol-induced neuromorphological damage at all stages of development. There are several reasons for using different animal models to estimate the brain damage caused by ethanol. The type and severity of the damage depend on the timing of the insult as well as the dose and peak blood ethanol concentration. In the course of development, all mammals go through a period of most rapid brain growth, called the "brain growth spurt" in humans this begins in the third trimester and the equivalent period in the rat is roughly the first 10 postnatal days with a peak at postnatal days 6 to 8. The third trimester is extremely vulnerable to exogenous insults. The present experiment used the rat during this vulnerable period as a model of the ethanol impediment on brain development. Use of this animal model enabled us to investigate the protective effects of drugs against brain growth impedi ment caused by ethanol. Recently, Burns et al. developed a new method to examine the toxic effects of ethanol on the brain using pups. They suggested that episodic ethanol exposure (6 g/kg) on a single day (postnatal day 6) causes permanent brain damage with properties similar to those induced by chronic ethanol exposure throughout the major portion of the growth spurt. They used oral administration of ethanol to observe the ethanol toxicity. However, the oral administration of relatively high ethanol concentrations had several disadvantages such as a large variation in effects and the appearance of cytotoxic effects on gastrointestinal functions. We therefore decided to examine ethanol's effect on pup brain growth when it was administered subcutaneously.

The root of Panax ginseng C. A. Mayer (ginseng) has been widely used as the principal material in many prescriptions in traditional Chinese medicine to alleviate and cure a variety of diseases. It has been reported that ginseng relieves various symptoms of degenerative diseases including amnesia and aging. Several saponins have been identified as main components in ginseng. Ginsenosides Rb₁ and Rd potentiated the effect of nerve growth factor (NGF) in organ cultures of chicken embryonic dorsal root ganglia and lumba sympathetic ganglia. Ginsenosides Rg₁ and Rb₁ had promoting effects on the survival of rat cerebral cortex neurons in cell cultures. A crude ginseng saponin had a proliferative effect on neurite extension and a protective effect on distortion of neurites due to cytochalasin B. These studies led us to assume that ginseng saponins, major constituents of ginseng root, affect the ethanol-induced toxicity in the CNS. In the present study, we examined the protective effects of ginseng extract and its constituents on impaired brain growth in neonatal rats exposed to ethanol.

MATERIALS AND METHODS

Animals Primiparous female Wistar rats (210—260 g) were obtained from Shimizu Laboratory Supplies (Kyoto, Japan). Adult male rats were placed with three females overnight, and pregnancies were timed from the presence of spermatozoa in a vaginal smear taken before 10.00 a.m. each day. The day of spermatoza appearance was designated as gestational day 0. The pregnant rats were randomly assigned to ethanol exposure and control groups, housed individually in plastic cages with sawdust bedding and given food and water ad libitum under controlled environmental conditions.

Procedure to Induce Brain Growth Impediment The animals were exposed to either ethanol or saline according to the schedule shown in Fig. 1. Gestational day 21 was defined as postnatal day 0, even if the length of gestation varied. The experiments were performed using litters of more than 10 pups. On postnatal day 3, the ten largest pups were culled from each litter. Eight of the ten rats were randomly used for experiments, and the two remaining rats were housed with the other rats to observe the conditions of their littermates. These two rats were not used for the experiments. Four to 6 groups of the 8 littermates (total 32—48 pups) were used for each
Fig. 1. Administration Schedule

The experiment except that to determine blood ethanol concentration (total 18 pups). On postnatal day 6, eight of the littermates were treated with 3 g/kg (s.c.) of a 20% (w/v) of absolute ethanol in water two times at an interval of 3 h. This ensured that the ethanol exposed groups received 6 g/kg body weight dose of ethanol on one day only. Other treatments were performed using the schedule described above. Control groups were treated with 100 μl/pup (s.c.) of saline. Pentobarbital groups were treated with 30 mg/kg (15 mg/kg × 2, s.c.) of pentobarbital. Pentobarbital and isolation groups were separated from dams for 15 h after 18.00 h. Ethanoltreated and control groups were placed in a cage at room temperature with dams. Isolation and pentobarbital groups were placed in a cage on a heated pad (27±3°C) to avoid a decrease in body temperature. All pups were weighed daily throughout postnatal days 6 to 14 and on postnatal day 20. On postnatal day 20, the pups were deeply anesthetized with ether and sacrificed by decapitation. Their brains were removed, and whole brain and cerebellar weight were measured. Blood ethanol concentrations were determined by head space gas chromatography. The blood samples were obtained 45 min, 24 and 48 h after the second 3 g/kg ethanol administration, when the pups were decapitated.

Preparation and Treatment of Samples Dried roots of Panax ginseng C. A. Mayer were extracted with 95% ethanol, and the extract diluted to a final concentration of 30% ethanol. After filtration, the filtrate was lyophilized, and the dried ginseng extract was submitted to column chromatography on MCI-gel CHP 20P (75—150 μm, Mitsubishi Kasei Co., Ltd.) using a stepwise gradient elution of 20% ethanol and 50% ethanol. Ginseng saponins were isolated and purified from 50% ethanol eluate according to the procedure of Sanada et al. Ginseng extract, 20% ethanol eluate and 50% ethanol eluate (saponin fraction) were dissolved in saline, and ginseng saponins were suspended in distilled water. Test samples were subcutaneously administered once a day for 5 consecutive days throughout postnatal days 7 to 11.

Statistical Analysis All values represent the mean ± standard error, and the results were statistically evaluated by Dunnett’s two-tailed test. The percentages of recovery of the treatment groups were determined by comparing mean cerebellar weight of each group (T) with those of the ethanol exposure group (E) and control group (C) as follows: \((T - E)/(C - E)) \times 100\).

RESULTS AND DISCUSSION

Animal Model of Ethanol-Induced Brain Growth Impediment The consequences of ethanol exposure during early postnatal development are relatively unknown. In the rodent, this period is comparable to the human third trimester, and is characterized by a brain growth spurt. Microcephaly is a particularly important morphological characteristic of the FAS, and the obvious consequence of that is a reduced size of the brain (microencephaly), which is a common finding following exposure to ethanol during the third trimester equivalent. Therefore, we established an animal model for ethanol-induced brain damage by modifying the method described by Burns et al.

Ethanol at a total dose of 6 g/kg (s.c.) significantly decreased the mean weight of brain, cerebral cortex and cerebellum on postnatal day 20 as shown in Fig. 2. The relative weights of brain, cerebral cortex and cerebellum in control groups were 3.70 ± 0.03 g, 2.13 ± 0.02 g and 430 ± 3.78 mg per 100 mg body weight (mean ± S.E.), respectively. The cerebellum (almost 30% less) seems to be particularly highly sensitive to postnatal ethanol administration, possibly because this region undergoes almost all of its growth during the postnatal period. These brain growth impediments were detected even at postnatal day 41. Burns et al. also confirmed the disproportionate reduction in cerebellar weight, altered balancing ability and a decreased number of cerebellar cells in both ethanol-exposed groups on postnatal days 17 and 70. This suggests that the ethanol-induced impediment of the brain growth is an irreversible phenomenon. As postnatal day 20 corresponds to the end of the brain growth spurt period, the brain weight was measured on this day in subsequent experiments. It has been reported that the concentration in the blood and not the injected dose was the crucial factor for ethanol’s adverse effects on brain growth. In the present study, mean ethanol concentrations detected in the blood after 45 min, 24 and 48 h of the second ethanol administration were 365, 102 and 39 mg/dl, respectively. High blood ethanol levels interfered with the pups’ ability to suck properly, resulting in a nutritional deficiency. To establish an animal model...
for impaired brain growth following exposure to ethanol, it is essential to separate the effect of ethanol as a drug from that of ethanol-induced malnutrition. Ethanol at the doses used in this study inhibited spontaneous activity including sucking behavior for as long as 15 h. Therefore, ethanol-induced effect on the brain weight was compared with the isolation group to determine whether or not suppression of feeding plays a role in ethanol-induced brain growth impediment. In contrast to the ethanol exposure group, no obvious decrease in brain weight was observed in the isolation group. This suggests that ethanol-induced reduction of the brain weight is not due to a suppression of feeding.

It is widely accepted that ethanol causes a general depression of the CNS. Accordingly, acute intoxication of 6 g/kg of ethanol administration makes pups stuporous and comatose. Impeded brain growth may therefore result from ethanol’s general depressive action on the CNS. To test this possibility, we examined the toxic effects of pentobarbital which can also produce potent depressive effects on the CNS. All the pups survived following treatment with ethanol (total 6 g/kg) or pentobarbital (total 30 mg/kg), but after pentobarbital administration at a total dose of 40 mg/kg, all rats (8 pups) died. The righting reflex was inhibited after the first and second administrations of ethanol; loss of this reflex after the second administration of ethanol lasted more than 6 h. Similar inhibition of the righting reflex was observed in pentobarbital treated groups. On postnatal day 6, pentobarbital (total 30 mg/kg) was administered in a similar manner as that used for ethanol, but it did not affect the postnatal brain maturity (Fig. 2). Therefore, it is not likely that a brain growth impediment was caused from ethanol’s effect as a CNS depressant.

**Effect of Extract and Constituents of Ginseng** Cerebellar weight is a good indication of the character of deficient growth on postnatal brain development. Figure 3 depicts the recovery percentage of the cerebellum that was examined by subcutaneous administration of ginseng extract (100 mg/kg), 20% ethanol eluate (200 mg/kg) and 50% ethanol eluate (10 mg/kg) against ethanol insult. Ginseng extract had a slight tendency to protect against cerebellar growth impediment, while both 20% and 50% ethanol eluates fractionated by chromatography on a column of MCI-gel CHP 20P were significantly effective. In particular, the 50% ethanol eluate exerted relatively high protection (35%) even at a dose of 10 mg/kg.

Percentages of body weight gain between postnatal days 6 and 20 are shown in Fig. 4. Linear increase in the mean body weight gain was observed in the control group, and the isolation group showed a similar increase. In the ethanol exposure group, mean body weight decreased the day (postnatal day 7) following ethanol exposure, but a linear increase was observed on postnatal days 8 to 20. In the group treated with ethanol and saponin fraction of ginseng extract, the body weight increased in a similar manner as did the ethanol exposure group.

On TLC analysis the 50% ethanol eluate showed the presence of ginseng sapogenin as the major component, but ginseng sapogenin was not detected in the 20% ethanol eluate. These results suggested that the protective activ-

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**Fig. 4. Effects of Isolation, Ethanol and Ethanol Plus Saponin on BodyWeights during Postnatal Days 6 to 20**

The ordinate indicates mean gain of body weight. Weight gain was calculated with the following equation, weight gain (%) = \( ((B W_f - B W_i) / B W_i) \times 100\), in which \( B W_i \) is body weight on postnatal day shown in the abscissa, and \( B W_f \) is body weight on postnatal day 6. Control: saline treated group; Isolation: pups were separated for 15 h. Ethanol: pups exposed to ethanol (total 6 g/kg). Ethanol + saponin: pups exposed to ethanol (total 6 g/kg) and 50% ethanol eluate (10 mg/kg/d × 5 d).

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**Fig. 5. Structures of Ginseng Saponins**

- **20(S)−protopanaxadiol glycosides**
  - Ginsenoside Rh1: Glc-Glc-H
  - Ginsenoside Rh2: Glc-Glc-H
  - Ginsenoside Re: Glc-Glc-H
  - Ginsenoside Rf: Glc-Glc-H

- **20(S)−protopanaxatriol glycosides**
  - Ginsenoside Re: H-Rha-Glc-O
  - Ginsenoside Rg3: H-Glc-Glc-O

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ity might be due to these ginseng saponins. Then, the 50% ethanol eluate (27.08 g) was subjected to column chromatography to give ginsenosides Rg1 (0.46 g), RF (0.59 g), Rd (2.66 g), Re (0.70 g), Rg3 (3.41 g), Rb2 (4.77 g) and Rb1 (5.49 g); the structure of each saponin is shown in Fig. 5.

Figure 6 shows recovery percentage from the application of 5 and 20 mg/kg ginseng saponins. Treatment with 5 mg/kg dose did not prevent cerebellar growth impendiment, however, administration of 20 mg/kg dose displayed marked efficiency. This provides some evidence that the brain growth impendiment suffered from ethanol can be regenerated with these saponins. However, the present study did not show any structure-activity relationships between 20(S)-protopanaxadiol and 20(S)-protopanaxatriol glycosides.

In the ethanol exposure group, decrease in the body weight was observed on the second day after the treatment. Saponin treatment did not have such an ethanol-induced effect on body weight. Linear increase in the weight was observed over the next 14 d in both groups. Saponin-induced protective action thus appears not to be caused by the body weight changes. It is plausible that ginseng saponins directly or indirectly interfere with the action of ethanol in the CNS.

Further study is necessary to determine the mechanism of the toxic effect of ethanol and the protective effect of saponin. In the present study, ethanol exposure induced marked reduction of the cerebellar growth. This may be due either to cell loss or the impaired growth of neuron fibers such as axons and dendrites, although we did not carry out histological examination of the cerebellum. Two major neuronal populations of cerebellum are Purkinje cells which are large cells formed prenatally, while granule cells are small cells are formed postnatally. Recent histological study has revealed that Purkinje cell loss resulted from combined pre- and postnatal ethanol exposure, but not from prenatal exposure alone.14)

Ethanol exposure on postnatal days 3 to 4 only resulted in a significant permanent reduction in the number of Purkinje cells, with no further reduction upon exposure throughout days 3 to 20 postnatally.15) Purkinje cell loss in lobule IX was not found after ethanol exposure from postnatal days 6 to 16.16) These data suggest that ethanol exposure in the rat during postnatal days 2 to 4 may be especially toxic to maturing Purkinje cells. Since ethanol was administered at postnatal day 6 in this study, ethanol-induced impaired growth of the cerebellum may not be due to the injury of Purkinje cells but rather due to that of other types of neurons in the cerebellum. It has been reported that ginseng saponins produce neuro-pharmacological effects such as the promotion of nerve fiber production induced by NGF,17) the prolongation of cortex neuron survival,17) and the accelerating action on neurite extension of cortex neurons as well as protective effects against neurite distortion. Those studies together with the present findings indicate that ginseng saponins may have neuroprotective functions against ethanol toxicity.

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