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Effect of Diets Supplemented with Pharbitis Seed Powder on Serum and Hepatic Lipid Levels, and Enzyme Activities of Rats Administered with Ethanol Chronically

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The levels of γ-aminobutyric acid (GABA) have been analyzed from pharbitis seeds by an AccQ-Tag amino acid analysis procedure. The GABA level of the pharbitis seeds was 125 nmole per gram fresh weight. To investigate the effects of pharbitis seed diets on serum and hepatic lipid levels, as well as enzyme activities of rats administered with ethanol chronically, Sprague-Dawley male rats were fed with either a AIN-76 diet (control), a control diet plus ethanol, a control plus pharbitis seed diet, or a control plus pharbitis seed diet plus ethanol for 30 days. Pharbitis seed diets decreased the serum total cholesterol, triglyceride, LDL-cholesterol, and γ-GTP levels that were increased by the chronic ethanol administration. In addition, pharbitis seed diets decreased the liver triglyceride and total lipid levels that were increased by the ethanol administration. However, ethanol metabolism was not retarded by the pharbitis seed supplemented diets. The present findings, plus previous data showing the differences in the effects of cabbage diets having a high or a low level of GABA on the lipid levels and the enzyme activities of rats (Cha and Oh [2000] J. Korean Soc. Food Sci. Nutr. 29, 500-505), raise the possibility that GABA in plants could have a nutraceutical role in the recovery of chronic alcohol-related diseases.

Keywords: Ethanol, GABA, Lipids, Pharbitis seeds.

Introduction

Pharbitis nil Choisy is a short-day plant that is grown throughout China, Japan, and Korea (Bensky and Gamble, 1993; Kim, 1996). Pharbitis seeds have some cathartic, renal, and antiparasitic effects (Bensky and Gamble, 1993). In the People's Republic of China and Korea, pharbitis seeds, usually combined with other herbs, have been used for the prevention and cure of liver diseases, such as fatty liver and liver cirrhosis (Kim *et al.*, 1978; Lee and Lee, 1986). Pharbitis seeds have been reported to contain pharbitic acid, glycosides, flavonoids, phenolic compounds and anthocyanin (Lu *et al.*, 1992; Bensky and Gamble, 1993; Saito *et al.*, 1993, 1994).

Studies have shown that liver is the principal organ responsible for ethanol metabolism (Lieber, 1991, 1994). The first step in the metabolism of ethanol is its oxidation to acetaldehyde. This conversion is carried out almost entirely in the liver. Many studies have shown that ethanol, as well as malnutrition that has been induced by ethanol, contributes to liver disease (Mezey, 1980; Lieber, 1991). Ethanol oxidation causes liver injury through a variety of mechanisms due to the nature of high chemical reactivity of the acetaldehyde produced (French, 1989; Lieber, 1991). The oxidation of ethanol in the liver increases the NADH/NAD ratio, which is responsible for many of the deleterious effects of ethanol, such as ketosis, hyperlipidemia, hyperuricemia, and fatty liver (Lieber, 1991, 1994).

γ-aminobutyric acid (GABA) is a ubiquitous nonprotein amino acid that is produced primarily from an α-decarboxylation of L-glutamic acid (Glu) catalyzed by the enzyme glutamate decarboxylase (GAD) (Satyanarayan and Nair, 1990; Choi *et al.*, 1998; Park *et al.*, 1999). GABA functions in animals as a major inhibitory neurotransmitter (Erlander and Tobin, 1991; Mody *et al.*, 1994; Sung *et al.*, 1999). GABA is involved in the regulation of cardiovascular functions, such as blood pressure and heart rate, and plays a role in the sensation of pain and anxiety (Krogsgaard-Larsen, 1989). Many neurological disorders, such as seizures, Parkinson's disease, stiff-man syndrome, and schizophrenia are related to alterations of the GABA and GAD levels in the brain (Krogsgaard-Larsen, 1989; Bao *et al.*, 1995). In alcoholics, remarkably low plasma GABA levels, and a low

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Table 1. Composition of basal and experimental diets[1)]

Ingredients	Groups[2)]			
	Control	Control + EtOH	Control + Pharbitis	Control + Pharbitis + EtOH
Casein	20.0	20.0	20.0	20.0
DL-methionine	0.3	0.3	0.3	0.3
Corn starch	15.0	15.0	10.0	10.0
Pharbitis seed powder	-	-	5.0	5.0
Sucrose	50.0	50.0	50.0	50.0
Fiber	5.0	5.0	5.0	5.0
Corn oil	5.0	5.0	5.0	5.0
AIN mineral mix	3.5	3.5	3.5	3.5
AIN vitamin mix	1.0	1.0	1.0	1.0
Choline bitartrate	0.2	0.2	0.2	0.2

[1)]All components are in units of g/100 g diet.
[2)]In +EtOH groups, 13%(v/v) ethanol (3 g/kg body weight) was administered for 30 days, as described in Materials and Methods.

expression of the GABA receptor in the brain, have been measured (Krogsgaard-Larsen, 1989; Morrow, 1997).

The presence of GABA in plants has been known for at least half a century (refs. in Satyanarayan and Nair, 1990). However, the role of GABA in plants is unclear. It has been suggested that this compound may have a role in pH regulation, in N storage, in plant development, and in the plant's defense against phytophagous insects (Bown and Shelp, 1997). Recently, the utilization of GABA as a functional plant component has received increased interest. In fact, there are several lines of evidence showing that plant extracts containing high levels of GABA are effective for blood pressure regulation (Omori et al., 1987; Nakagawa and Onota, 1996), and for the recovery of alcohol-related symptoms (Nakagawa and Onota, 1996; Cha and Oh, 2000).

In the present work, we analyzed the level of GABA in pharbitis seeds, and tested the effect of diets supplemented with pharbitis seeds on blood-ethanol concentrations in rats. We also analyzed the effects of the diets on plasma and hepatic lipid levels, and enzyme activities of rats administered with ethanol chronically. Then, the possible roles of pharbitis seed GABA in the effects were discussed in relation to the present findings.

Materials and Methods

Materials GABA, DL-methionine, corn starch, sucrose, fiber, corn oil, choline bitartrate were purchased from Sigma Chemical Co. (St. Louis, USA). The AIN-76 vitamin and mineral mix were from Teklard (USA). Casein was from Cottee (Australia). All other chemicals were of the highest commercial grade available. Pharbitis seeds were supplied from Yoo's Oriental Medical Clinic (Iksan, Chonbuk).

Analysis of GABA and free amino acids Free amino acids, including GABA, were extracted from the pharbitis seeds using a modified procedure of Baum *et al.* (1996). Pharbitis seeds were frozen in liquid nitrogen, and ground to a fine powder with a mortar and pestle. The pharbitis seed powder (~200 mg) was transferred to a 800 μl mixture of water : chloroform : methanol (3 : 5 : 12, v/v/v). The homogenate was centrifuged at 12,000 × g for 15 min, and the aquous fraction of supernatant was collected. The pellet was dissolved by adding 200 μl chloroform and 400 μl water, and centrifuged at the same conditions as described above. The aquous phase was then collected. The collected fractions were combined, dried in a freeze dryer, and dissolved in water. Samples were passed through 0.45 μm PVDF filters (Millipore, Bedford, USA), and analyzed by using an AccQ-Tag amino acid analysis system (Waters, Milford, USA).

Animal and diets Sprague-Dawley rats that weighed about 100g were supplied from Daehan Biolink Inc. (Eumsung, Chungbuk, Korea). They were housed individually in stainless steel cages with a randomized complete block design, in a temperature of 23 ± 1°C, humidity of 53 ± 2%, and a light controlled room with a 12-hr light-dark cycle. The animals were fed normal chow (Jeil-jedang Suwon, Korea) for 1 week, then randomly divided into 4 groups: control diet, control diet plus ethanol, control plus pharbitis seed diet, and control plus pharbitis seed diet plus ethanol group. Each of the 4 groups had 6 rats. The compositions of the experimental diets are shown in Table 1. Pharbitis seeds were ground to powder of 100 mesh. Experimental diets and water were provided *ad libitum*, and changed each morning between 9 : 00~10 : 00. The rats were orally administered 13% (v/v) ethanol (3g/kg body weight) once a day for 4 weeks in ethanol-treated groups, and distilled water of the same volume in ethanol-nontreated groups (Rhew and Sachan, 1986). Body weight was measured weekly.

After feeding the 4 diets (Table 1) for 4 weeks, a single dose (3 g/kg body weight) of ethanol (13%, v/v, solution) was orally administered, and serial blood samples were collected from the tail vein. Blood was collected at 30, 60, 120, 180, 240 and 360 min post-ethanol administration, and blood-ethanol concentrations were determined by an enzymatic method (Bernt and Gutmann, 1974).

Prior to sacrificing the rats, food was withheld from their cages for 12 hours. Each rat was anesthetized by the inhalation of ether.

Blood samples were collected from the heart with non-heparinized syringes, and incubated on ice water 1 hr. Serum was separated from the collected blood by centrifugation at 1,100 × g for 15 min at 4°C, and kept at -70°C until analyses. The liver was removed, rinsed with a phosphate buffered saline solution, wiped with a paper towel, and kept at -70°C.

Analysis of lipids and enzyme activities Serum total cholesterol levels were measured by using commercial kits from Asan Pharm. Co. (Seoul, Korea), based on the cholesterol oxidase method (Allain *et al.*, 1974). The HDL-cholesterol fraction was prepared by the dextran sulfate-Mg^{++} method (Warnick *et al.*, 1978). The HDL-cholesterol level was analyzed enzymatically using commercial kits from Asan Pharm. Co. (Seoul, Korea). The LDL-cholesterol level was calculated by the Friedwald method (Friedwald *et al.*, 1972). Serum triglyceride levels were measured by the lipase-glycerol phosphate method (McGowan *et al.*, 1983) using commercial kits (Asan Pharm. Co., Seoul, Korea). Serum glutamate-oxaloacetate transaminase (GOT), glutamate-pyruvate transaminase (GPT) and γ-gutamyltransferase (γ-GTP) levels were determined using commercial kits (Asan Pharm. Co., Seoul, Korea). Liver lipids were extracted from liver tissues according to the method of Folch *et al.* (1983), and the total lipid levels were measured by the sulfo-phospho-vanillin method (Frings and Dunn, 1970) using commercial kits (Kokusai Pharm. Co., Kobe, Japan). Triglyceride levels were measured by the lipase-glycerol phosphate method, as described previously.

Statistical analysis Data from individual experiments were expressed as the means ± standard deviation. All statistical analyses were performed on a statistical analysis system (SAS) program, and significant difference between mean values were determined by using Duncan's multiple range test (Duncan, 1993) that $p<0.05$ was judged to be statistically significant.

Results and Discussion

GABA and free amino acid contents in pharbitis seeds By using the AccQ-Tag amino acid analysis method (Cohen and Michaud, 1993), levels of GABA and free amino acids were measured. As shown in Table 2, the GABA level in pharbitis seeds was 125 nmole per gram fresh weight. The levels of GABA from other plant sources, such as cabbage leaves (Oh *et al.*, 2000) and ginger roots (unpublished data), were 14 and 49 nmoles per gram fresh weights, respectively. The data show that pharbitis seeds contain relatively high levels of GABA compared with the cabbage and ginger samples. The levels of free amino acids in pharbitis seeds were in order of Ala>Gly>Ser>Asp>Glu>Tyr>Arg>Pro>Phe>Val>Thr>Ile>Leu>His>Met.

Table 2. Free amino acid and GABA levels in pharbitis seed[1)]

	Pharbitis seed (nmole/g fresh weight)
Asp	145(±5)
Ser	157(±13)
Glu	135(±7)
Gly	207(±20)
His	21(±2)
Arg	119(±10)
Thr	56(±3)
Ala	211(±19)
GABA	125(±15)
Pro	108(±16)
Tyr	121(±12)
Val	58(±4)
Met	7(±2)
Ile	37(±5)
Leu	31(±7)
Phe	67(±7)
Total	1,605(±147)

[1)]Free amino acids containing GABA were extracted from pharbitis seeds and analyzed as described in Materials and Methods. The data represents the mean of three determinations with standard deviation of the mean.

Body weight gain Body weight at sacrifice was decreased significantly in the group administered with both control diet and ethanol compared with the control diet group. However, the significant body weight reduction due to the chronic ethanol administration was not observed in the group fed the pharbitis-supplemented diet (Table 3). Previously, it was shown that over consumption of ethanol leads to a decrease of body weight (Lieber, 1994; Oh *et al.*, 1999). This is because ethanol lacks essential nutrients, other than calories, which

Table 3. Body weight gain of animals for experimental period

Groups	Treatment period(week)				
	0	1	2	3	4
Control	140.6±7.8[1)a]	171.4±5.2[2)a]	210.8± 7.4[a]	224.4±5.0[a]	269.6±12.2[a]
Control + EtOH	138.0±3.6[a]	165.2±5.8[ab]	179.0±14.7[b]	189.2±6.3[b]	240.8± 7.6[b]
Control + Pharbitis	133.9±6.2[a]	160.8±5.2[b]	196.3±12.1[ab]	226.3±5.9[a]	258.6±10.8[ab]
Control + Pharbitis + EtOH	135.3±8.2[a]	158.0±7.1[b]	192.7± 6.9[ab]	220.2±8.7[a]	255.7±13.2[ab]

[1)]The values represent the gram of weight with the standard deviation of the mean of 6 rats per group.
[2)]Different superscripts in the same columns indicate significant differences ($p<0.05$) among groups by Duncan's multiple range test.

Table 4. Effects of pharbitis diets on lipid levels of serum and liver

Groups	S-TChol	S-TG	S-HDLc	S-LDLc	Liver-TG	Liver-TL
	(mg/dL)				(mg/g wet weight)	
Control	91.4±11.7[1)b]	62.9±16.0 [2)b]	50.1±7.8[b]	16.0±6.1[ab]	17.3±0.8[b]	54.3±17.0[b]
Control + EtOH	111.8± 9.3[a]	99.2±12.8[a]	68.5±7.4[a]	22.0±5.9[a]	22.6±4.3[a]	103.7±32.0[a]
Control + Pharbitis	77.4±20.1[b]	43.4± 7.7[b]	33.0±9.2[b]	11.6±2.5[b]	14.6±1.2[b]	45.7± 4.0[b]
Control + Pharbitis + EtOH	77.8±14.5[b]	51.5±12.4[b]	34.2±3.3[b]	15.9±5.9[ab]	15.5±4.3[ab]	43.2±18.0[b]

[1)]The values represent the mean with standard deviation of the mean of 6 rats per group.
[2)]Different superscripts in the same columns indicate significant differences ($p<0.05$) among groups by Duncan's multiple range test.
S, serum; HDLc, HDL-cholesterol; LDLc, LDL-cholesterol; TChol, total cholesterol; TG, triglyceride; TL, total lipid

Table 5. Effect of pharbitis diets on blood-ethanol concentrations (mg/dl) in rats

Minutes PEA	Groups			
	Control	Control + EtOH	Control + Pharbitis	Control + Pharbitis + EtOH
30	159.7±40.8[1)]	158.0±21.6	143.1±74.4	154.6±86.7
60	205.1±36.0	205.3±39.0	202.1±52.8	187.5±73.3
120	209.6±33.9	199.7±33.0	177.2±48.8	181.6±28.0
180	221.3±17.6[2)a]	188.3±28.8[a]	182.9±37.9[a]	133.4±24.7[b]
240	207.9±26.9[a]	166.8±22.0[b]	120.5±25.0[bc]	90.9±31.0[c]
360	172.2±35.0[a]	86.4±14.4[b]	76.4±18.4[b]	48.9± 8.8[c]

[1)]The values represent the mean with standard deviation of the mean of 6 rats per group.
[2)]Different superscripts in the same rows indicate significant differences ($p<0.05$) among groups by Duncan's multiple range test.
PEA, post-ethanol administration.

results in primary malnutrition by displacing other nutrients in the diet (Scheig, 1970). Secondary malnutrition may be the result from either maldigestion, or malabsorption of nutrients caused by gastrointestinal complications (Scheig, 1970; Mezey, 1980). These malnutritions can induce deficiency of virtually all of the nutrients (Lieber, 1991).

Serum and hepatic lipid levels Generally, the measurement of the LDL-cholesterol level in the blood is considered in determining the malfunction of the lipoprotein metabolism, because the LDL-cholesterol level has shown a direct proportional correlation to both coronary heart disease and atherosclerosis (Goldstein and Brown, 1983; Steinberg and Witztum, 1990). In our study, the pharbitis seed diet decreased the serum total cholesterol, triglyceride, HDL-cholesterol and LDL-cholesterol levels, which were increased with the chronic ethanol administration (Table 4). Also, the pharbitis seed diet decreased the liver triglyceride and total lipid levels, which were increased with the chronic ethanol administration (Table 4).

Since GABA showed an inhibition effect on the peptic output in anaesthetized rats (Goel *et al.*, 1996), the decrease in serum and hepatic lipid levels might be due to a retarded absorption and metabolism of ethanol by the pharbitis seed GABA. To test whether or not the pharbitis supplemented diets retard ethanol metabolism, thereby causing an elevation of blood-ethanol concentrations (BEC), we determined BEC in the 4 groups of rats. As shown in Table 5, the BEC was significantly lower in the pharbitis supplemented diet groups than in the control group at and beyond 240 min post-ethanol administration (PEA). The BEC in the rats of the control diet plus ethanol group was not significantly different from that of the control group rats, except that it was lower at 360 min PEA. Previously, it was shown that GABA was unable to inhibit rat liver alcohol dehydrogenase at the NAD concentration of 100 μM (Sachan and Cha, 1994). The results allow us to conclude that the ethanol clearance, and hence ethanol metabolism, is not retarded by pharbitis seeds containing the level of GABA in Table 2.

Serum enzyme activities It has been shown that ethanol, as well as malnutrition that was induced by ethanol, contributes to liver disease, resulting in a high level of hepatic enzymes in serum (Lieber, 1991, 1994). The pharbitis seed diet significantly decreased the blood γ-GTP level that was increased due to the ethanol administration (Table 6). However, serum GPT levels were not significantly different among the groups (Table 6). Several studies have shown that diets supplemented with plants, or the administration of plant extracts, have an effect on the reduction of the serum enzyme activities that were increased by ethanol (Kim and Lee, 1996; Oh *et al.*, 1999) or CCl_4 (Lee *et al.*, 1993; Bae *et al.*, 1997). The administration of pharbitis seed extracts decreases the level of the serum enzyme activities that were increased by

Table 6. Effects of the pharbitis diets on the levels of several enzymes in serum

Enzyme	Groups			
	Control	Control + EtOH	Control + pharbitis	Control + pharbitis + EtOH
GOT(mU/ml)	53.4±4.2[1)b]	76.8±6.3 [2)a]	61.2±5.8[b]	70.9±8.2[ab]
GPT(mU/ml)	26.8±2.4[a]	28.7±3.0[a]	28.3±4.2[a]	26.7±6.5[a]
γ-GTP(mU/ml)	6.0±1.5[ab]	10.6±3.3[a]	4.9±1.7[b]	4.1±1.1[b]

[1)]The values represent the mean with standard deviation of the mean of 6 rats per group.
[2)]Different superscripts in the same rows indicate significant differences ($p<0.05$) among groups by Duncan's multiple range test.

CCl_4 (Kim *et al.*, 1978; Lee and Lee, 1986). Recently, we found that a diet supplemented with cabbage roots containing a higher level of GABA than the control cabbage decreased plasma γ-GTP level that was increased by the ethanol administration (Cha and Oh, 2000). Future studies with GABA supplemented diets, as well as pharbitis seeds that have a higher concentration of GABA (i.e., germinated seeds), may provide further insight into the roles of GABA in recovery effects. Also, further studies to elucidate the elevation of GABA levels by the pharbitis seeds in rat tissues such as brain and serum, and the interaction of pharbitis seed components with GABA synthesizing enzyme or degrading enzyme may provide further insights into approaches for the nutraceutical application of pharbitis seeds.

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