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<Original Article>

Hangover relieving effect of Sanghwang mushroom mycelium cultured in germinated buckwheat

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Abstract: The present study was performed to evaluate the hangover relieving effect of germinated buckwheat (GB) and Sanghwang mushroom mycelium cultured in GB (SGB). Both GB and SGB showed 1,1-diphenyl-2-picrylhydrazyl radical scavenging activities and significantly increased ($p < 0.001$) aldehyde dehydrogenase (ALDH) activities; up to 140% increase at concentrations of 16 μL/mL. Locomotor activity test results from alcohol-SGB and alcohol-GB groups showed improved motor activities over that of the alcohol-water group at 90 min post-administration. Both alcohol-GB and alcohol-SGB groups had significantly reduced ($p < 0.001$) alcohol (40.02 ± 33.38 μg/mL, 66.01 ± 22.04 μg/mL, respectively) and aldehyde (5.72 ± 0.47 μg/mL, 6.72 ± 1.70 μg/mL, respectively) concentrations in blood compared to those in the alcohol-water group (199.75 ± 33.83 μg/mL, 50.43 ± 13.88 μg/mL, respectively) at 90 min post-administration. Based on cDNA microarray analysis, expressions of ALDH genes ALDH1a7 and ALDH18a1 and cytochrome P450 (CY450) gene CYP4a30b were upregulated in the alcohol-GB and alcohol-SGB groups compared to levels in the control group. Overall, the results suggest that both GB and SGB have hangover relieving effects by reducing blood acetaldehyde levels. The molecular mechanisms may involve ALDH activation and upregulated expression of alcohol metabolism-related genes such as ALDH and CYP450.

Keywords: Sanghwang mushroom mycelium, aldehyde concentration, germinated buckwheat, hangover relieving effect, locomotor activity test

Introduction

Alcohol is closely related to human life, and drinking alcohol causes many negative effects on our society and economic growth [1]. Heavy alcohol drinking is associated with many social problems and consequently many studies have focused on addressing alcoholism and relieving hangover [16]. Alcohol and its metabolites damage the liver and cause irreversible liver disease by inducing inflammatory responses during alcohol metabolism [4]. Because alcohol is a molecule capable of permeating all the tissues of the body, it can affect most vital functions. Alcohol metabolism in hepatocytes occurs via three major pathways: alcohol dehydrogenase (ADH) pathway, microsomal ethanol oxidizing system, and catalase [24]. Alcohol is metabolized into acetaldehyde and acetic acid by several liver enzymes including ADH and acetaldehyde dehydrogenase (ALDH), respectively [29]. ALDH oxidizes more than 90% of acetaldehyde produced from alcohol, transforming acetaldehyde into acetate in a reaction coupled with nicotinamide adenine dinucleotide (NAD^+) reduction [9]. Acetaldehyde is a primary metabolite produced by oxidizing alcohol which causes hangover symptoms including hot flush, increased pulse, nausea, and vomiting [3, 28]. In addition, acetaldehyde causes functional modulation of proteins [20], glutathione (GSH) depletion, free radical-mediated toxicity and lipid peroxidation [24]. Previous study observed that chronic drinking of reduces ALDH activity [19]. Alcohol intake inhibits antioxidants, and induces oxidative stress by producing oxygen radicals due to increased nicotinamide adenine dinucleotide (NADH) oxidase activity [23], and it causes hepatic cell injury [14].

To investigate functional materials which improve antioxidant activity and alcohol decomposition, a variety of medicinal plants and natural materials have been studied. In particular, various physiological activities of Sanghwang mushroom have been reported including its antioxidant effect [31], anticancer effect [5], and immune-stimulating effect [18]. In addition, germinated buckwheat (GB) has been reported for its antioxidant effect [8], anti-hypertensive and anti-hyperlipidemic effects [21]. In this study, hangover relieving effects

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of GB and Sanghwang mushroom mycelium cultivated on GB (SGB) were evaluated through ADH and ALDH activity test, locomotor activity test, blood alcohol and acetaldehyde concentration measurements of mice pre-treated with alcohol. In addition, the differential gene expression patterns in mice liver were analyzed through cDNA microarray analysis to observe the effects of GB or SGB on gene expression related to alcohol metabolism.

Materials and Methods

Sample preparation

Sanghwang mushroom mycelium was cultivated on germinated buckwheat for two weeks, and was roasted for 48 hours at 65°C. Samples and distilled water were mixed in a ratio of 1 : 20, and were distilled above 95°C for 3 hours. Extracts were filtered using filter papers, and concentrated using a vacuum concentrator (55 rpm, 40–45°C). The concentrations of samples were set to 10 Brix which corresponds to about 10 percent of sugar derivatives in solution, and were used for the experiments.

Experimental animals

Male BALB/c mice (6 weeks old) were supplied by Orient Bio (Korea). Mice were housed at 24 ± 1°C and 50-55% humidity under a 12 h light/12 h dark cycle for two weeks prior to experiments. All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Jeju National University (ACUCC; Approval No. 2016-0007).

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities

The antioxidant activities of GB and SGB extracts were determined by measuring the DPPH free radical scavenging ability of GB and SGB extracts. GB or SGB extracts were dissolved in DMSO separately to make a concentration series (final concentration 0, 2, 10, 40 80, 120 μg/mL). 50 μL of each sample was mixed with 950 μL of 0.2 mM DPPH (Sigma, USA) solution. The mixture was incubated for 30 min at room temperature in dark condition, and absorbance was measured at 517 nm using spectrophotometer (Mecasys, Korea). Antioxidant activity was calculated as follows; Antioxidant activity (%) = {1 − (Absorbance of a group added to sample / Absorbance of a group non-added to sample)} × 100.

ADH/ALDH activities

ADH activity was measured by using an ethanol quantification assay kit (Megazyme, Ireland) following manufacturer's instructions. Briefly, distilled water 1 mL, buffer (pH 9.0) plus sodium azide (0.02% w/v) 0.1 mL, NAD^+ solution 0.1 mL, and 20% ethanol 10 μL were mixed, and then GB and SGB extracts (1, 2, 4, 8, 16 μL/mL of 10 Brix samples) were added separately. Solutions were incubated for 2 min at room temperature, and absorbance (A1) was measured at 340 nm. Then 10 μL (1 assay unit) of ADH was added to each mixture, mixtures were incubated for 5 min, and increased absorbance (A2) due to NADH accumulation was measured. ADH activity was represented by relative enzyme activity (%) to the control group based on the changed absorbance (A2–A1). ALDH activity was also measured by using acetaldehyde quantification assay kit (Megazyme) following manufacturer's protocol.

Locomotor activity test

The locomotor activity cage (Ugo Basile, Italy) was used to observe the activity of mice. The mice were divided into four groups (10 mice each group), and the three groups were treated with water, GB or SGB (4 mL/kg) 30 minutes after alcohol (2 mL/kg) treatment. The control group was sham-treated with water at 30 minutes following initial administration of water. Each group of mice was kept in locomotor activity cage (43 × 43 × 30 (h) cm^3) and their activities in 10-minute period were recorded for 990 min by monitoring infrared beams hit numbers.

Blood alcohol and acetaldehyde concentrations

The mice were divided into four groups as described above. Then, blood was harvested from each mouse at 90 min post-administration, and blood alcohol and acetaldehyde concentrations were measured. Blood samples were drawn and left at room temperature for 20 min. Serum was separated by centrifugation at 900 × g for 10 min and the resulting product was stored at −70°C until use. Blood alcohol concentrations were measured using ethanol quantification assay kit (Megazyme) according to manufacturer's protocol. Briefly, serum 50 μL, distilled water 1 mL, buffer (pH 9.0) plus sodium azide (0.02% w/v) 0.1 mL, NAD^+ solution 0.1 mL and 25 μL (1 assay unit) of ALDH solution were mixed. The solution was incubated for 2 min at room temperature and absorbance (A1) was measured at 340 nm. After measuring absorbance (A1), 10 μL (1 assay unit) of ADH was added to the mixture, and the mixture was incubated for 5 min and increased absorbance (A2) due to NADH accumulation was measured. Blood alcohol concentrations were calculated using the extinction coefficient of NADH at 340 nm based on the changed absorbance (A2–A1). Blood acetaldehyde concentration was also measured by using acetaldehyde quantification assay kit (Megazyme) according to the method of Megazyme protocol.

cDNA microarray analysis

Total RNA was isolated from mice livers (harvested 90 minutes after pretreatment with any intervention) using the Easy-Blue RNA extraction kit (iNtRON Biotechnology, Korea). Transcription of dsDNA was performed by adding the transcription master mix to the dsDNA reaction samples and incubating at 40°C for 2 hours. Amplified and labeled cRNA

was purified on cRNA Cleanup Module (Agilent Technology) according to the manufacturer's protocol. Labeled cRNA target was quantified using ND-1000 spectrophotometer (NanoDrop Technologies, USA). After checking labeling efficiency, fragmentation of cRNA was performed by adding 10× blocking agent and 25× fragmentation buffer and incubating at 60°C for 30 min. The fragmented cRNA was resuspended with 2× hybridization buffer and directly pipetted onto assembled Agilent's Mouse Oligo Microarray (44 K). The arrays hybridized at 65°C for 17 h using Agilent Hybridization oven (Agilent Technology). The hybridized microarrays were washed as the manufacturer's washing protocol (Agilent Technology). The hybridized images were scanned using Agilent's DNA microarray scanner and quantified with Feature Extraction Software (Agilent Technology). Functional annotations of genes were performed according to Gene Ontology Consortium [2] by GeneSpringGX 7.3. Gene classification was based on searches done by Medline databases (National Center for Biotechnology Information, USA).

Statistical Analysis

The one-way analysis of variance (ANOVA) and the Dunnett's test (SPSS 12.0; SPSS, USA) were used to evaluate differences between groups. Results are expressed using descriptive statistics, *i.e.*, the mean ± SE.

Results

DPPH radical scavenging activities

DPPH radical scavenging activities were measured to observe the antioxidant activities of GB and SGB. The dose response curves of the DPPH radical scavenging activities of GB and SGB were compared over a range from 2 to 120 μL/mL. At a concentration of 120 μL/mL, the antioxidant activity of GB and SGB was increased to 30% and 20% respectively (Fig. 1).

ADH/ALDH activities

To observe the ADH or ALDH activation ability of GB or SGB, the total enzyme activities were measured in the presence of GB or SGB in vitro. ADH activities were slightly increased up to 110% by GB or SGB, with no observed significance (Fig. 2A). ALDH activities were increased by GB or SGB in a dose-dependent manner. GB showed a significantly increased ($p < 0.001$) ALDH activity up to 140% at a concentration of 16 μL/mL. In addition, SGB showed significantly increased ($p < 0.001$) ALDH activity up to 139% at a concentration of 16 μL/mL (Fig. 2B).

Locomotor activity test

Locomotor activity tests were performed to observe the travel distances of mice treated with alcohol-GB or alcohol-SGB. Mice in the control (water-water) group maintained higher locomotor activity compared to the treated groups throughout the time. The alcohol-water group showed markedly decreased locomotor activity at 90 min following treatment. In contrast, the alcohol-GB and alcohol-SGB groups showed more improved locomotor activities compared with the alcohol-water group (Fig. 3).

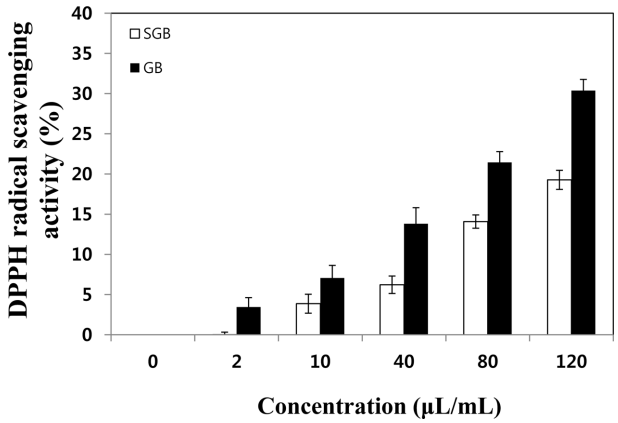

Fig. 1. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity followed by different concentrations of germinated buckwheat (GB) and Sanghwang mushroom mycelium cultured by GB (SGB). Values are the means ± SE of radical scavenging activities (n = 5).

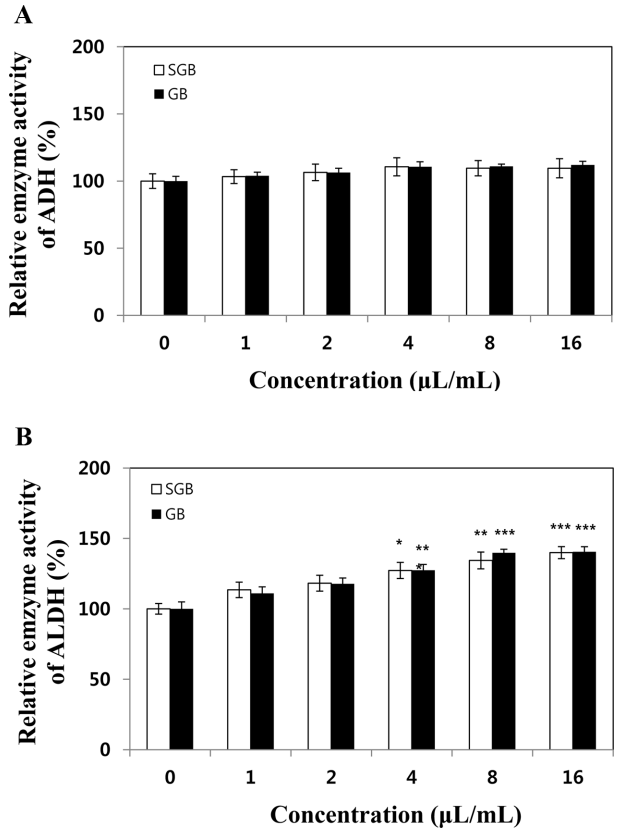

Fig. 2. ADH activation by GB and SGB (A). ALDH activation by GB and SGB (B). The concentration of GB and SGB were set to 10 Brix after extraction, and were used for the experiment. Values are the means ± SE of relative enzyme activity of control (n = 5). $^{*}p < 0.05$, $^{**}p < 0.005$ and $^{***}p < 0.001$ compared to control.

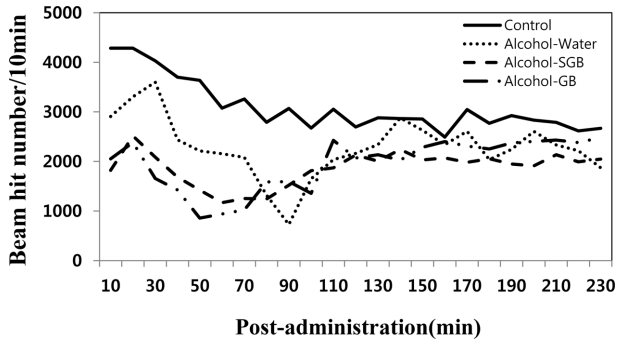

Fig. 3. Locomotor activity of mice administered with GB and SGB. Traveled (ambulatory) distances were measured using a locomotor activity cage with 43 cm square and 30 cm height walls. Mice were divided into four groups (10 mice each) and three groups were administered with water, GB or SGB at 30 min after ethanol (2 mL/kg) treatment. Control group was administered with water at 30 min after pre-administration of water. Tests were performed immediately after each administration. Values are means of number of infra-red beams hit during a 10 min period in each group.

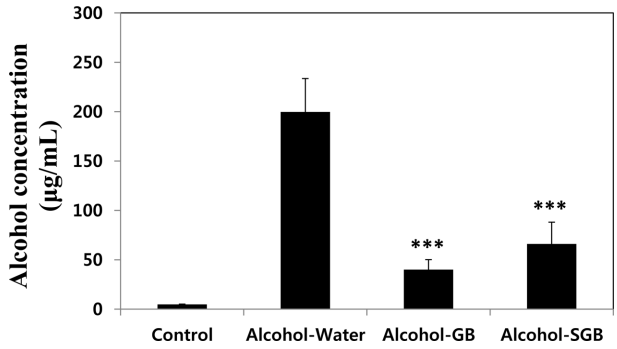

Fig. 4. Alcohol concentrations in the blood of mice administered with GB and SGB. Mice were divided into four groups (10 mice each) and three groups were administered with water (4 mL/kg), GB or SGB at 30 min after alcohol (2 mL/kg) treatment. Control group was administered with water at 30 min after pre-administration of water. Alcohol concentrations were measured at 90 min after each administration. Values are the means ± SE of alcohol concentrations. (***$p < 0.001$) compared to alcohol-water group.

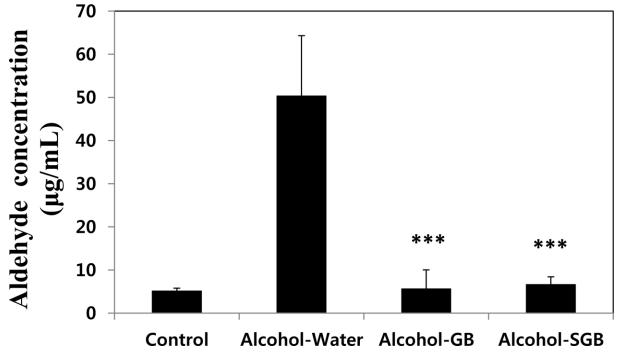

Fig. 5. Aldehyde concentrations in the blood of mice administered with GB and SGB. Mice were divided into four groups (10 mice each) and three groups were administered with water (4 mL/kg), GB or SGB at 30 min after alcohol (2 mL/kg) treatment. Control group was administered with water at 30 min after pre-administration of water. Aldehyde concentrations were measured at 90 min after each administration. Values are the means ± SE of aldehyde concentrations. (***$p < 0.001$) compared to alcohol-water group.

Blood alcohol and acetaldehyde concentrations

Blood alcohol and acetaldehyde alcohol concentrations were measured at 90 min after treatment. The alcohol-water group showed a significantly increased blood alcohol level (199.75 ± 33.83 μg/mL) compared with the control group (4.78 ± 0.32 μg/mL). Alcohol-SGB group showed a significantly decreased ($p < 0.001$) blood alcohol concentration (66.01 ± 22.04 μg/mL) compared to the alcohol-water group. In addition, the alcohol-GB group showed a significantly reduced blood alcohol concentration (40.02 ± 33.38 μg/mL) compared to alcohol-water group (Fig. 4). Alcohol-water group showed a significantly increased blood acetaldehyde concentration (50.43 ± 13.88 μg/mL) compared with the control group (5.21 ± 0.56 μg/mL). In contrast, the alcohol-SGB group showed a significantly reduced ($p < 0.001$) blood aldehyde concentration (6.72 ± 1.7 μg/mL) compared with the alcohol-water group. In addition, alcohol-GB group showed significantly reduced ($p < 0.001$) blood acetaldehyde concentration (5.72 ± 0.47 μg/mL) compared to the alcohol-water group (Fig. 5).

cDNA microarray analysis

cDNA microarray analysis was performed to observe the differential gene expression in mice liver by alcohol, GB and SGB. Based on the results of cDNA microarray analysis, among 39,429 genes in mouse liver, 523 genes were up-regulated and 1,565 genes were down-regulated in alcohol-GB group compared to alcohol-water group (Table 1). A total of 543 genes and 1,109 genes were up- or down-regulated respectively, in the alcohol-SGB group compared with the alcohol-water group (Table 2). Among the genes involved in alcohol metabolic enzymes, ADH4 and ADHfe genes in ADH family were up-regulated by 1.40 and 1.32 fold respectively, and ALDH1a7 and ALDH18a1 genes in ALDH family were up-regulated by 2.37 and 1.64 fold in the alcohol-SGB group compared to the alcohol-water group (Table 3). Gene CYP4a30b in cytochrome P450 (CYP450) family was up-regulated by 2.30 and 2.17 fold in alcohol-SGB and alcohol-GB groups respectively (Table 3). In contrast, ALDH1a3 and ALDH3a1 genes in ALDH family were down-regulated by 0.49 and 0.09 fold respectively. CYP2s1, CYP27b1, CYP2j13, CYP24a1, and CYP2j11 genes in CYP450 family were down-regulated by 0.47, 0.32, 0.14, 0.08 and 0.04 fold respectively in alcohol-SGB group compared to alcohol-water group (Table 4). ALDH1a3 and ALDH3a1 genes in ALDH family were down-regulated by 0.33 and 0.07 fold, and CYP2s1, CYP27b1, CYP2j13, CYP24a1 and CYP2j11 genes in CYP450 family

Table 1. Differential gene expression of alcohol- germinated buckwheat group compared to the alcohol-water group

	Total	Aging	Apoptotic process	Cell cycle	Cell death	Cell different	Cell migration	Cell prolifer	Immune response	Inflamma-tory response	Neuro-gene-sis	Secretion
Gene number	39,429	393	1,194	1,506	1,281	4,657	985	773	1,150	536	2,126	636
% of Total	100.0	1.0	3.0	3.8	3.2	11.8	2.5	2.0	2.9	1.4	5.4	1.6
Up significant	523	12	28	18	29	75	29	11	43	36	31	10
% of Up significant	1.3	3.1	2.3	1.2	2.3	1.6	2.9	1.4	3.7	6.7	1.5	1.6
Down significant	1,565	23	43	36	47	258	63	26	53	26	106	43
% of Down significant	4.0	5.9	3.6	2.4	3.7	5.5	6.4	3.4	4.6	4.9	5.0	6.8

Table 2. Differential gene expression of alcohol-Sanghwang mushroom mycelium cultured in GB group compared to the alcohol-water group

	Total	Aging	Apoptotic process	Cell cycle	Cell death	Cell different	Cell migration	Cell prolifer	Immune response	Inflamma-tory response	Neuro-gene-sis	Secretion
Gene number	39,429	393	1,194	1,506	1,281	4,657	985	773	1,150	536	2,126	636
% of Total	100.0	1.0	3.0	3.8	3.2	11.8	2.5	2.0	2.9	1.4	5.4	1.6
Up significant	543	0	9	16	10	54	11	10	14	5	24	3
% of Up significant	1.4	0.0	0.8	1.1	0.8	1.2	1.1	1.3	1.2	0.9	1.1	0.5
Down significant	1,109	12	34	28	37	178	44	22	35	24	73	28
% of Down significant	2.8	3.1	2.8	1.9	2.9	3.8	4.5	2.8	3.0	4.5	3.4	4.4

Table 3. Up-regulated genes related to the alcohol metabolism by SGB or GB

	ADH		ALDH		Cytochrome P450	
	Gene name	Fold changed	Gene name	Fold changed	Gene name	Fold changed
Alcohol-SGB/Alcohol-water	ADH4 ADHfe1	1.40 1.32	ALDH1a7 ALDH18a1 ALDH1b1	2.37 1.64 1.53	CYP3a44 CYP2c55 CYP51 CYP4a30b	4.60 3.98 2.43 2.30
Alcohol-GB/Alcohol-water					CYP4a30b	2.17

ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase.

Table 4. Down-regulated genes related to the alcohol metabolism by SGB or GB

	ADH		ALDH		Cytochrome P450	
	Gene name	Fold changed	Gene name	Fold changed	Gene name	Fold changed
Alcohol-SGB/Alcohol-water	–	–	ALDH1a3 ALDH3a1	0.49 0.09	CYP2s1 CYP4a14 CYP4a31 CYP2b13 CYP27b1 CYP4a32 CYP4a10 CYP2j13 CYP24a1 CYP2j11	0.47 0.43 0.41 0.38 0.32 0.26 0.19 0.14 0.08 0.04
Alcohol-GB/Alcohol-water	–	–	ALDH1a3 ALDH3a1	0.33 0.07	CYP2s1 CYP27b1 CYP2j13 CYP2c65 CYP2c66 CYP11a1 CYP24a1 CYP2j11 CYP11b1	0.37 0.32 0.21 0.19 0.14 0.09 0.07 0.04 0.02

were down-regulated by 0.37, 0.32, 0.21, 0.07 and 0.04 fold in alcohol-GB group compared to the alcohol-water group (Table 4). Based on the results of cDNA microarray analysis, the molecular mechanisms of hangover-relieving may involve up-regulated expression of genes involved in alcohol metabolism, including ALDH and CYP450 genes.

Discussion

In this study we evaluated the hangover-relieving effects of GB and SGB. Both GB and SGB showed DPPH radical scavenging activities, and showed significantly increased ALDH activities in vitro. In addition, both GB and SGB improved locomotor activities of mice pre-treated with alcohol, and showed a significantly reduced alcohol and aldehyde concentrations in blood compared to alcohol-water group at 90 min post-administration. In addition, both GB and SGB showed the differential expression of genes related to alcohol metabolism in ALDH and CYP450 gene families. Overall, the results suggest that both GB and SGB have hangover-relieving effects by reducing acetaldehyde levels in the blood. The molecular mechanisms probably involve through up-regulation of ALDH, CYP450 and other genes involved in the metabolism of alcohol.

Enzymatic and non-enzymatic mechanisms exist to protect cells from the internal or external reactive oxygen species supplied by sources such as diet, vitamins, and polyphenols [22, 11]. Therefore, enhancing protective mechanisms may protect cells against the consequences of oxidative mechanisms delaying the onset of certain conditions including aging and cancer [10, 30]. Accordingly, many studies are focused on relieving hangover, using antioxidant components derived

from natural products which have no side-effect [27, 33]. In the present study, maximal antioxidant activity of GB and SGB was observed at 30% and 20% respectively, which are lower than previous observations [13, 31]. The results indicate that the extract of Sanghwang mushroom mycelium which was cultivated on germinated buckwheat has a lower DPPH radical scavenging activity compared to the extracts from either Sanghwang mushroom or Sanghwang mushroom mycelium which was cultivated on different media.

As observed previously [13], the present study showed that markedly decreased locomotor activity at 90 min in the alcohol-water group, whereas the alcohol-GB and alcohol-SGB groups showed more improved locomotor activities at 90 min. However, unexpectedly, the alcohol-GB and alcohol-SGB groups showed lower locomotor activities than the alcohol-water group until 80 min. The results indicate that the media, germinated buckwheat, might affect to a lower locomotor activity of mice at earlier stage of hangover. Further studies are necessary to evaluate this phenomenon.

The present study showed that ALDH activity was increased up to 140% by GB or SGB, whereas ADH activity was slightly increased with no observed significance. ADH and ALDH are the principal enzymes responsible for ethanol metabolism. Previous studies showed that ADH and ALDH activities altered by alcohol administration were normalized by dose of *Pueraria thunbergiana* extracts supplement [12]. ADH4 is a key member of the ADH family. It mainly contributes to liver ADH activity and may account for as much as 40% of the total ethanol oxidation rate at intoxicating levels of alcohol [32]. The present study showed that the ADH4 gene was up-regulated by 1.4 fold in the alcohol-SGB group compared to the alcohol-water group. The result suggests that hangover relief in the alcohol-SGB group might partially result from increased expression of the ADH4 gene by the active intervention of SGB. Interestingly, the present study observed that the subfamilies of ALDH1 including ALDH1a7, ALDH1b1 which play a role in detoxification of aldehydes were up-regulated by SGB [26]. In addition, ALDH3a1, which catalyzes the $NAD(P)^+$-dependent oxidation of aromatic and medium chain aldehydes [17], was down-regulated by GB or SGB. Further study is necessary to investigate the role of these subfamilies in alcohol metabolism.

CYP450 is an enzyme superfamily that catalyzes the oxidation of a wide variety of endogenous and exogenous substrates. The present study observed that the CYP4a30b gene, which is known to synthesize 20-hydroxyeicosatetraenoic acid [6], was up-regulated by either GB or SGB. In addition, CYP2c55 gene which catalyzes synthesis of 19-HETE, an arachidonic acid metabolite [15] and the CYP450 sterol 14á-demethylase (CYP51) which is an essential enzyme in the biosynthesis of sterols [7] were up-regulated by SGB. In contrast, CYP2j family members who produce *cis*-epoxyeicosatrienoic acids, mid-chain *cis*–transconjugated dienols and ω-terminal alcohols [25] were down-regulated either by GB or SGB. In future studies, the role of CYP2j family members in alcohol metabolism should be elucidated.

In the present study, the levels of acetaldehyde in the blood of mice at 90 min pre-administration were inversely proportional to the motor activities of mice, which suggest that improved motor activities of mice were the result of reduced blood acetaldehyde concentration by GB and SGB. cDNA microarray analysis showed that differentially expressed genes associated with alcohol metabolism included ADH, ALDH and CYP450 genes. Overall, the results suggest that both GB and SGB have hangover-relieving effects by reducing acetaldehyde levels in the blood through not only activating ALDH but also up-regulating the expression of the genes associated with alcohol metabolism including ALDH and CYP450. Even though both GB and SGB have hangover-relieving effects for mice, further studies are needed to extrapolate the effectiveness of this topical application to hangover-relieving for human.

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