Food Sci. Biotechnol. Vol. 15, No. 2, pp. 238 ~ 243 (2006)

Food Science and Biotechnology

Hepatoprotective Effects of Waxy Brown Rice Fermented with *Agrocybe cylindracea*

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Abstract The methanol extract of waxy brown rice fermented with *Agrocybe cylindracea* was prepared. The extract was then freeze dried and fed to rats at the level of 0.5, 1.0, 2.0 g/kg body weight for 14 days, followed by the treatment with carbon tetrachloride for three consecutive days to induce hepatotoxicity. After sacrificing the rats, the enzyme activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), and γ-glutamyl transpeptidase (γ-GTP) in serum was determined. Biochemical analysis on serum for albumin, total protein, triglyceride, and total as well as HDL-cholesterol were carried out along with a histopathological study of liver tissues. Based on these data, we suggest that the waxy brown rice cultured with *A. cylindracea* may exert hepatoprotective activity against hepatotoxicity caused by chemicals such as carbon tetrachloride.

Keywords: *Agrocybe cylindracea*, ALT, AST, CCl_4, lipid peroxide, histological examination

Introduction

Today, the human body is exposed to many chemical substances including carbon tetrachloride, acetaminophen, adriamycin, and alcohol which might be a cause of liver damage (1). Carbon tetrachloride is one of the most widely used hepatic toxins for the experimental induction of hepatic fibrosis and cirrhosis in laboratory animals (2). The CCl_4-induced fibrosis in experimental animals resembled the human fibrosis in some aspects of morphology and pathophysiology (3, 4). It is now accepted that administration of CCl_4 results in liver injury characterized by increases in serum liver enzymes (5) and histological manifestations of inflammation and fibrosis (6, 7).

There are several reports on the hepatoprotective activities from various mushrooms. Mushrooms that are reported to have such effect are *Ganoderma lucidum*, *Phellinus linteus*, *Lentinus edodes*, *Hericium erinaceus*, and *Agaricus blazei* (8-13). Most of these studies, however, have been carried out using the fruiting body or mycelium, rather than food products derived from these mushrooms.

The functional foods and medicines produced by the use of medicinal mushroom strains are on the domestic market at present. The functional rice, marketed since 1999, is produced from waxy brown rice on which *Phellinus* sp. are cultured by solid state fermentation technique. The biological functionality of the product was also reported (14-16).

Agrocybe cylindracea is one of the favorite edible mushrooms in Japan, which has recently been introduced into commercial production in Korea. Very few studies, therefore, on the biological functionality of *A. cylindracea* have been reported. The hepatoprotective activity of the mushroom rice produced by the fermentation of waxy brown rice with *A. cylindracea* was investigated in this study. The methanol extract of the mushroom rice was prepared and fed to experimental animals and the indicated biochemical and histopathological studies were performed.

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Received November 12, 2005; accepted February 2, 2006

Materials and Methods

Fungal strain *Agrocybe cylindracea* was obtained from the National Agricultural Science and Technology Institute, Korea. The strain was maintained in MYG slant (malt extract, 1%; yeast extract, 0.4%; dextrose, 0.4%; agar, 1.5 %). The strain was cultured at 28°C in MYG agar for 7 days prior to use.

Culturing and sample preparation The agar piece was cut out with a cork borer of 5 mm diameter and used to inoculate starter cultures. After 14 days of shaking culture, the mycelium was homogenized and used to inoculate the waxy brown rice that has been soaked, sterilized and cooled. The inoculated rice was then fermented for 14 days at 28°C. The solid state fermented rice was extracted with methanol, filtered, concentrated by a vacuum evaporator, and freeze dried (brown *A. cylindracea*, BAC). For comparison, unfermented waxy brown rice was also extracted with methanol and prepared the same way as the sample (waxy brown rice sample, BR). The samples were kept at -70°C for experimental use.

Experimental animals Male Sprague-Dawley rats (5 weeks old) were acclimated for 5 days to the experimental environment on a 12/12 day:night cycle with normal solid feed. The rats were divided into 6 groups, with 6 rats in each group. Sufficient feed and water were supplied while

maintaining temperature and relative humidity at 28°C and 50±10% respectively.

Sample feeding and carbon tetrachloride treatment The normal group (NOR) was fed with 1 g/day/kg (body weight) distilled water in place of sample apart from the normal diet, followed by feeding 1 mL/day/kg (body weight) olive oil without carbon tetrachloride for the last three days. The control group (CON) was fed the same way as normal group except that the olive oil of the last 3 days contained the same amount of carbon tetrachloride, 1mL/kg body weight, to induce hepatotoxicity. The waxy brown rice group (BR) was fed and the hepatotoxicity was induced the same way as CON except that 1 g/day/kg (body weight) BR specimens were fed for 14 days with 1 g/day/kg BR sample apart from normal feed. BR samples was the freeze-dried methanol extract of waxy brown rice and this was resuspended in distilled water prior to feeding. Functional waxy brown rice groups (BAC) were fed and the hepatotoxicity was induced as the same way as BR group except that 0.5 g (BAC I), 1.0 g (BAC II), and 2.0 g (BAC III) of BAC sample per day per kg body weight were fed, respectively, in place of BR sample. Vitamin B and C complex was used as a positive control and fed the same way as the other groups in place of distilled water or samples. Table 1 summarizes the experimental groups and treatments used in this study.

Blood collection and separation of serum The animals fasted for 12 hr prior to the administration of ether anesthesia, followed by the extraction of blood from the main abdominal artery. The organs were extracted after washing out the blood with ice cold 0.15 M KCl buffer solution. Kidney, spleen, heart, and lung were washed with physiological saline and dried by absorbing water with filter paper. The weights of organs were determined and expressed as gram per 100 g body weight. The blood was centrifuged at 2,250×g for 20 min at 4°C in order to obtain serum.

Biochemical analyses of serum Test kits (Asan, Korea) for aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity were used and the enzyme activity was expressed according to the Retiman-Frankel method (17). The alkaline phosphatase (ALP) activity was measured using the King-King method (18). Lactate dehydrogenase (LDH) activity was measured using the lactate substrate method, and γ-glutamyl transpeptidase (γ-GTP) activity was measured using the 5-amino salicylic acid method. The albumin content of serum was measured using the B.C.G method and total protein was measured using the Biuret method using standard test kits (Asan). HDL-cholesterol, triglyceride, and total cholesterol were determined by enzymatic assay using standard test kits (Asan).

Lipid peroxide content in liver tissue The liver tissue was homogenized with 4 volumes of 0.1 M potassium phosphate buffer (pH 7.4) at 4°C using a homogenizer (Ultra-turrax TM 750; Kuala Lumpur, Malaysia). This was then centrifuged at 600×g for 10 min to obtain supernatant. The lipid peroxide formed by carbon tetrachloride treatment was determined by the method of Ohkawa *et al.* (19).

Electron microscopy of liver tissues Liver tissues were cut into 1 mm^3 cubes right after dissection and pre-fixed in 2.5% glutaraldehyde solution that was prepared with 0.1 M phosphate buffer (PBS, pH 7.4) for 2.5 hr. The pre-fixed tissues were washed three times at 4°C at 10 min intervals with the same buffer solution. These were again fixed for 2 hr in 1% osmium tetraoxide that was prepared with PBS. The fixed tissues were dehydrated by using ethanol at stepwise increasing concentrations from 50 to 100%, followed by finishing up with propylene oxide. These were then formatted with epoxy and cut into 1ìm semi-thin sections with an ultra microtome. The sections were observed after simple dyeing with 0.5% toluene blue in order to choose the sites to be observed with the electron microscope. The selected sites were then cut into

Table 1. Experimental groups

Group	Sample Dose (g/kg)	Treatments
NOR[1)]	-	Basal diet
CON[2)]	-	Basal diet + Carbon tetrachloride
BR[3)]	1	Basal diet + BRM + Carbon tetrachloride
BAC I[4)]	0.5	Basal diet + ACBRM + Carbon tetrachloride
BAC II[5)]	1	Basal diet + ACBRM + Carbon tetrachloride
BAC III[6)]	2	Basal diet + ACBRM + Carbon tetrachloride
VTC[7)]	1	Basal diet + VT + Carbon tetrachloride

[1)] Normal group.
[2)] Rats were treated with CCl_4 after 2 weeks of feeding with basal diet.
[3)] Rats were fed with basal diet containing methanol extract of waxy brown rice (BRM), followed by CCl_4 treatment.
[4)] Rats were fed with basal diet containing methanol extract of waxy brown rice fermented with *Agrocybe cylindracea* (ACBRM), 0.5 g/day/kg body weight, followed by CCl_4 treatment.
[5)] Rats were fed with basal diet containing methanol extract of waxy brown rice fermented with *A. cylindracea* (ACBRM), 1 g/day/kg body weight, followed by CCl_4 treatment.
[6)] Rats were fed with basal diet containing methanol extract of waxy brown rice fermented with *A. cylindracea* (ACBRM), 2 g/day/kg body weight, followed by CCl_4 treatment.
[7)] Rats were fed with vitamin B·C complex (VT), followed by CCl_4 treatment.

ultra thin sections and double dyed with uranyl acetate and lead citrate, then these sections were viewed using transmission electron microscopy(H-600; Hitachi Ltd., Tokyo, Japan).

Statistical analysis The data obtained were analyzed using SPSS for Windows Version 10.0 and the results were reported as mean ± standard deviation. The statistical significance was tested by Duncan's multiple range test of one-way ANOVA with SPSS program.

Results and Discussion

Effects of BAC on body and organ weight There was a statistically significant reduction in body weight of CON groups in comparison with NOR groups when CON groups were subjected to hepatotoxicity induction by carbon tetrachloride without receiving sample preparations of functional rice (Table 2). The BAC groups, on the contrary, showed no significant difference in body weight compared to NOR groups after hepatotoxicity induction. The organ weights, particularly liver weight, appeared to be affected by the carbon tetrachloride treatment (Table 3). Statistically significant increases in the weights of liver, kidney, and heart of CON rats were observed, while no significant increase in the organ weights of BAC groups was observed (Table 3). The weight gain in liver is attributed to the damage of the cellular membrane which, in turn, results in the accumulation of fat in liver tissue (20). The weights of other organs such as kidney and heart, also showed significant increase in the CCl_4 treated rats. This may be an indication that hepatotoxins do not solely affect liver.

Table 2. Body weight change in rats

Group[1)]	Initial weight (g)	Final weight (g)	Daily increase (g)
NOR	[2)]167.00±1.90^{b}[3)]	271.85±3.67^{a}	7.48±0.32^{a}
CON	167.17±2.86^{b}	254.93±1.47^{d}	6.26±0.17^{c}
BR	163.00±1.26^{c}	253.80±5.22^{d}	6.48±0.37^{bc}
BAC I	166.87±0.42^{b}	262.80±9.37^{bcd}	6.85±0.71^{abc}
BAC II	169.48±0.44^{a}	269.37±8.99^{ab}	7.13±0.65^{ab}
BAC III	164.53±1.10^{c}	260.20±11.45^{cd}	6.83±0.83^{abc}
VTC	164.00±1.10^{c}	268.08±2.85^{abc}	7.43±0.29^{a}

[1)]Groups are the same as Table 1.
[2)]The values are mean±SD (n=6).
[3)]The values followed by the same letter are not significantly different (p<0.05).

AST and ALT activity Hepatic cells participate in a variety of metabolic activities and contain a host of enzymes. AST and ALT are both produced by the liver and are required to metabolize amino acids. If injury involves organelles such as mitochondria (21), soluble enzymes such as AST, normally located in the organelle, will be also released. The elevated activities of AST and ALT in serum are indicatives of the cellular leakage and loss of the functional integrity of cell membranes in the liver (22). Serum AST and ALT activities of experimental animals are shown in Table 4. These two enzymes are known to be the most reliable indicators of liver damage. The AST activity of CON rats increased dramatically, more than 80%, in comparison with NOR rats, indicating the hepatotoxicity of the carbon tetrachloride treatment. Even though the BAC II and BAC III groups showed higher AST and ALT activities than in NOR groups, they showed significantly lower AST and ALT activities compared to CON groups. This suggests the possibility of protective effects of the sample prepared from the functional rice against the hepatotoxicity of carbon tetrachloride. Moreover, the hepatoprotective effects appeared to be dose dependent, which lends further support to our hypothesis that BAC contains hepatoprotective properties. In order to determine if the hepatoprotective effects come from waxy brown rice itself, the BR sample was tested along with BAC samples. Table 4 shows that the waxy brown rice itself exerted some hepatoprotective effect (20% less AST activity than CON), but this effect was considerably weaker than that of the BAC groups. The effect of BR on ALT activity (Table 4) was similar to the effect of BR on AST activity. These observations also lend support to our observations the hepatoprotective effect of BAC in rats.

Table 3. Internal organ weights of rats

Group[1)]	Relative weight (%)[3)]				
	Liver	Kidney	Spleen	Lung	Heart
NOR	3.81±0.14^{c}[2,4)]	0.80±0.02^{b}	0.25±0.02^{ab}	0.47±0.03^{b}	0.37±0.02^{b}
CON	4.45±0.29^{a}	0.87±0.04^{a}	0.26±0.02^{ab}	0.55±0.04^{a}	0.49±0.08^{a}
BR	4.36±0.17^{a}	0.84±0.03^{ab}	0.28±0.03^{ab}	0.53±0.04^{a}	0.43±0.05^{b}
BAC I	4.11±0.22^{cd}	0.81±0.04^{b}	0.25±0.02^{b}	0.47±0.05^{b}	0.39±0.05^{b}
BAC II	3.96±0.14^{d}	0.80±0.05^{b}	0.28±0.03^{a}	0.46±0.03^{b}	0.37±0.01^{b}
BAC III	3.94±0.30^{bc}	0.80±0.05^{b}	0.29±0.02^{a}	0.47±0.04^{b}	0.37±0.04^{b}
VTC	3.94±0.11^{bc}	0.80±0.05^{b}	0.28±0.02^{ab}	0.45±0.02^{b}	0.37±0.03^{b}

[1)]Groups are the same as Table 1.
[2)]The values are mean±SD (n=6).
[3)]Relative weight (%) = internal organ weight/body weight × 100.
[4)]The values followed by the same letter are not significantly different (p<0.05).

ALP, LDH, and γ-GTP activity ALP, LDH, and γ-GTP activity are often studied along with AST and ALT as indicators for hepatotoxicity because activities of these enzymes also increase when liver damage occurs. As shown in Table 4, both LDH and γ-GTP activities of BAC groups showed statistically the same level as the NOR rats while the activities of CON rats increased considerably in comparison with NOR. The ALP activity test also demonstrates the hepatoprotective effects of BAC samples by suppressing the activity increase in CCl_4 treated animals.

BAC effects on serum albumin and total protein The decreased protein synthesis due to liver damage caused by CCl_4 has also been reported by Jeong *et al.* (23) and Cho *et al.* (24). The serum albumin play a role as a cellular protein resource and any liver damage might result in lowered albumin content in serum (25). The total protein content of serum is also considered as an indicative value for liver trouble and nutritional deficiency. The albumin and total protein content in CON decreased considerably in comparison with NOR while those of BAC groups were maintained at nearly the same level as NOR (Table 5). These results lend further support to the existence of hepatoprotective properties in BAC.

BAC effects on HDL-cholesterol, total cholesterol, and triglyceride content in rat liver HDL cholesterol is known to stimulate the excretion of cholesterol and therefore is in inverse proportion to hyperlipemia. Low levels of HDL-cholesterol in the serum of liver cirrhosis patients is caused by decreased liver function (26). The HDL-cholesterol content of all the BAC sample groups were significantly higher than those of CON even though they were lower compared to the NOR group (Table 5). This observation may be the result of the increased synthesis of fatty acids induced by CCl_4 administration. CCl_4 also may have caused the decreased release of hepatic lipoprotein (27). The high level of HDL in BAC treated rats could be an indirect indication of hepatoprotective effect against CCl_4 toxicity.

BAC effects on lipid peroxide content in rat liver Malondialdehyde (MDA) content, one of the index substances for lipid oxidation (28), appeared to be significantly lower in livers obtained from in all sample groups compared with the CON group. Like the other parameters discussed above, MDA content of the BAC II and BAC III groups was significantly lower than of the CON group (Table 6). Lipid peroxidation is the result of increased oxidative stress and free radical and this in turn

Table 4. Effect of MeOH extract from waxy brown rice fermented with *Agrocybe cylindracea* on the serum enzyme activities of experimental animals

Group[1)]	AST (Karmen unit)	ALT (Karmen unit)	ALP (K-A unit)	LDH (Wroblewski unit)	γ-GTP (mU/mL)
NOR	92.22±3.06$^{e2,3)}$	42.14±5.30^{e}	31.44±4.12^{d}	237.79±26.90^{c}	8.38±0.60^{d}
CON	166.65±10.93^{a}	138.57±11.69^{a}	70.49±0.99^{a}	493.41±47.04^{a}	30.90±1.94^{a}
BR	135.37±3.92^{b}	123.41±4.57^{b}	65.91±3.43^{a}	451.94±27.99^{a}	25.46±3.24^{b}
BAC I	118.15±6.28^{c}	99.52±10.40^{c}	51.64±7.31^{b}	318.17±55.32^{b}	11.70±2.40^{c}
BAC II	110.09±6.19^{cd}	93.89±18.87^{cd}	47.29±9.13^{bc}	284.18±53.78^{bc}	9.84±0.94^{cd}
BAC III	107.04±8.80^{d}	92.38±4.15^{cd}	45.81±6.69^{bc}	247.74±49.27^{c}	8.77±0.62^{d}
VTC	97.69±6.28^{e}	84.92±1.19^{d}	42.07±1.28^{c}	240.35±37.92^{c}	9.04±1.49^{d}

[1)]Groups are the same as Table 1.
[2)]The values are mean±SD (n=6).
[3)]The values followed by the same letter are not significantly different ($p<0.05$).

Table 5. Effect of MeOH extract from waxy brown rice fermented with *Agrocybe cylindracea* on the serum components of experimental animals

Group[1)]	Albumin (g/dL)	Total Protein (g/dL)	HDL-cholesterol (mg/dl)	Triglyceride (mg/dl)	Total cholesterol (mg/dl)
NOR	3.92±0.15$^{a2,3)}$	5.50±0.72^{a}	49.20±10.65^{a}	30.79±8.58^{c}	38.96±3.17^{d}
CON	1.65±0.15^{d}	3.70±0.49^{b}	28.03±0.99^{e}	62.63±1.91^{a}	69.95±24.61^{a}
BR	1.79±0.21^{d}	4.15±0.17^{b}	29.30±1.71^{de}	49.47±2.38^{b}	61.70±1.20^{ab}
BAC I	2.98±0.12^{c}	5.11±0.11^{a}	34.29±2.34^{cd}	44.81±3.46^{b}	54.16±3.28^{bc}
BAC II	3.29±0.10^{b}	5.21±0.08^{a}	38.06±1.63^{bc}	35.19±3.58^{c}	45.72±1.77^{cd}
BAC III	3.42±0.14^{b}	5.27±0.16^{a}	39.49±0.92^{bc}	32.56±5.92^{c}	42.64±3.35^{cd}
VTC	3.84±0.12^{a}	5.42±0.62^{a}	40.76±6.39^{b}	32.03±2.02^{c}	41.22±2.45^{d}

[1)]Groups are the same as Table 1.
[2)]The values are mean±SD (n=6).
[3)]The values followed by the same letter are not significantly different ($p<0.05$).

Table 6. Liver lipid peroxide content in rats

Group[1)]	MDA (n mole/g of tissue)
NOR	[2)]21.07±0.81[e3)]
CON	48.89±1.46[a]
BR	43.42±0.68[b]
BAC I	31.07±3.23[c]
BAC II	29.22±1.78[c]
BAC III	26.32±2.09[d]
VTC	21.64±0.94[e]

[1)]Groups are the same as Table 1.
[2)]The values are mean±SD (n=6)
[3)]The values followed by the same letter are not significantly different ($p<0.05$).

is known to be one of the major causes of toxic liver injury. Miyazawa *et al.* (29) postulated that phosphatidylcholine hydroperoxide is deeply engaged in cellular damage of liver. The lower level of MDA in BAC treated rats could be inferred as indirect hepatoprotective effect against CCl_4 toxicity.

Histopathological evidence for hepatoprotective properties of BAC Carbon tetrachloride is one of the most widely employed hepatic toxins for the induction of hepatic fibrosis and cirrhosis in experimental animals since the morphology and pathophysiology caused by CCl_4 in animals resemble those occurring in humans (3, 4).

Therefore, the histological examination of liver sections can provide further support for the hepatoprotective effects of the sample preparation fed to experimental animals. Liver sections of NOR rats showed normal hepatic cells with well-preserved cytoplasm, a prominent nucleus and well-preserved cellular organelles such as mitochondria, Golgi complex, endoplasmic reticulum, and ribosomes (Fig. 1 A), while those of CON which were exposed to CCl_4 without the sample feeding clearly showed cell damage. The normal architecture of the liver was completely damaged when exposed to CCl_4, where cells demonstrated a highly damaged endoplasmic reticulum, cell vacuolation, and degenerate nuclei (9) (Fig. 1 B). On the other hand, the liver cells of the animals which received BAC maintained their cellular integrity such that these cells appear nearly the same as normal, untreated cells, and this effect was dose dependent (Fig. 1 D, E, F).

In conclusion, the methanol extract of waxy brown rice fermented with *A. cylindracea* exhibited a protective effect against the liver damage caused by CCl_4 when experimental animals were fed orally prior to CCl_4 exposure. This suggests that fermented rice could be a potential food production.

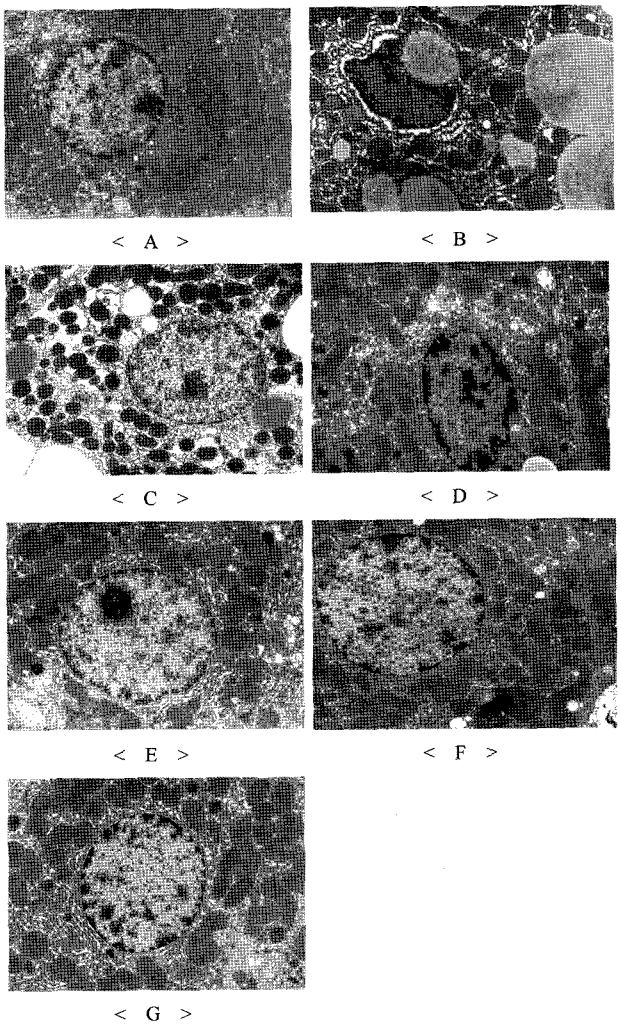

Fig. 1. Transmission electron micrographs (TEM) of rat hepatocytes. A, NOR group; B, CON group; C, BR group; D, BAC I group; E, BAC II group; F, BAC III group; G, VTC group.

Acknowledgments

This research was supported by the Yeungnam University research grants in 2004.

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