Food Sci. Biotechnol. Vol. 15, No. 1, pp. 122 ~ 127 (2006)

Food Science and Biotechnology

Fermented *Chaga* Mushroom (*Inonotus obliquus*) Effects on Hypolipidemia and Hepatoprotection in Otsuka Long-Evans Tokushima Fatty (OLETF) Rats

Jae-Young Cha, Bang-Sil Jun, Ki-Soo Yoo[1], Jong-Ryeal Hahm[2] and Young-Su Cho[1]*

Department of Biotechnology, College of Natural Resources and Life Science, Dong-A University, Busan 604-714, Korea
[1]Department of Anatomy, College of Medicine, Dong-A University, Busan 604-714, Korea
[2]Department of Internal Medicine, College of Medicine and Gyeongsang Institute of Health Science, Gyeongsang National University, Jinju, Gyeongnam 660-701, Korea

Abstract The effects of fermented *chaga* mushroom (*Inonotus obliquus*) powder on the lipid concentrations and the activities of liver marker enzymes of serum in genetically diabetic Otsuka Long-Evans Tokushima fatty (OLETF) rats were investigated. Rats were fed a semisynthetic diet supplemented with 50 g/kg *chaga* mushroom powder (CM) or 50 g/kg fermented *chaga* mushroom powder (FCM) for 8 weeks (26 to 34 weeks of age). Nondiabetic Long-Evans Tokushima Otsuka (LETO) rats were used as age-matched nondiabetic control animals. Water consumption was significantly higher in the OLETF control than the LETO rats. Water consumption in the FCM-fed OLETF rats tended to be less than in both the OLETF control and CM-fed OLETF rats. Serum concentrations of triglycerides and total cholesterol were significantly higher in the OLETF control rats than in the LETO rats while within the OLETF rat groups, the consumption of FCM resulted in a significantly lower serum triglyceride concentration and slightly lowered serum total cholesterol concentration when compared to the OLETF control and CM-fed rats. The alanine aminotransferase (ALT) activity was significantly higher in the OLETF control than in the LETO rats, but this difference was significantly reduced compared to the CM-fed rats and essentially no difference in the ALT levels was observed between the LETO and OLETF-FCM rats. This observation suggests an adaptive effect of the fermented *chaga* mushroom in liver function. Livers of the LETO rats showed no histopathological changes, whereas those of the OLETF control rats were characterized by many fat depositions in the central zone of the hepatocytes. The livers of the OLETF CM-fed rats showed less fatty changes compared to the OLETF control rats and fat deposition in the hepatocytes was nearly absent. These results suggest that orally ingested fermented *chaga* mushroom has a potential beneficial effect on the complications known to occur in the obesity-related non-insulin dependent diabetes mellitus (NIDDM) OLETF rat.

Keywords: *chaga* mushroom (*Inonotus obliquus*), OLETF rats, hypolipidemia, hepatoprotective effect

Introduction

Hyperlipidemia is a major risk factor associated with lifestyle-related diseases, obesity, arteriosclerosis and hypertension, and much attention has focused on improving serum lipids through the intake of natural diets (1, 2). The hypolipidemic effects of edible mushrooms, such as *Garnoderma lucidum* (3), *Pleurotus ostreatus* (4), *Lentinus edodes* (5), *Flammulina velutipes* (5), and various mushroom mixtures (6) have been reported to decrease serum lipid concentrations in hyperlipidemic and diabetic rat models. Most of the findings on the hypolipidemic properties were obtained from either whole mushroom powders (6) or water-soluble fraction of the mushrooms (3). Edible mushrooms are considered to be a dietary adjunct for the prevention of arteriosclerosis due to an ability to lower serum cholesterol and triglyceride concentrations, an effect attributed to an abundance of polysaccharides, polyphenolic compounds, fibers, lectins, glucans, and glycoprotein (1, 2, 7). Polysaccharides are perhaps the best known of these compounds and the most potent biologically active component of mushrooms with hypolipidemic effects in diabetic and hyperlipidemic animal models (8, 9). In addition, mushroom polysaccharides have also been reported to have antitumor, antioxidant and hypoglycemic properties (10, 11). The oligosaccharides consist primarily of di- and trisaccharides, derived from the hydrolysis of the beta-glucans e.g. the edible mushroom *Agaricus blazei* hydrolyzed by the endo-β (1→6)-glucanase of *Bacillus megaterium* (12). These compounds demonstrated anti-hyperglycemic, anti-hypercholesterolemic, and anti-arteriosclerotic activities in a diabetic rat model (12).

Several conditions, including obesity, type 2 diabetes mellitus or insulin resistance, hyperlipidemia, and other metabolic disorders have been known to cause hepatic injury in rats (2, 13). The activities of the liver marker enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum have been identified as risk factors to the development and the progression of hepatic injury (2, 14, 15). The edible mushrooms and their native constituents reportedly have a protective effect against both acute and chronic hepatic injury (7, 8). Polysaccharides and polyphenolic compounds have been found to be the major naturally occurring hepatoprotective mushroom constituents in extracts from edible medicinal mushrooms (13, 16, 17); total polyphenolic compounds in mostly edible mushrooms contained 0.48-1.28% (dry

*Corresponding author: Tel: 82-51-200-7586; Fax: 82-51-200-7505
E-mail: choys@dau.ac.kr
Received October 24, 2005; accepted November 19, 2005

weight) (18-22).

The *chaga* mushroom has been reported to contain polysaccharides and polyphenolic compounds with various biological and medicinal properties. The fruiting bodies of *Inonotus obliquus,* a member of the *Heterobasidine* family and commonly known as '*chaga* mushroom', have been traditionally used to treat gastrointestinal cancers, cardiovascular disease, and diabetes in Russia, Poland, and most Baltic countries (10, 11, 23-25). Many steroids and polyphenolic compounds (e.g. triterpenoids, lanosterol, inotodiol, trametenolic acids, hispoln, and hispidin from *Inonotus* sclerotia) have been identified as having hypotensive and antioxidative effects and properties (25, 26). A previous study that a water extract of *chaga* mushroom when fermented by *Bacillus* WRD-2 bacteria, had a cytotoxic effect on the HCT-15 colon carcinoma and AGS gastric carcinoma cell lines (19). In addition, fermented *chaga* mushroom reportedly to has a hypoglycemic effect in the type 1 streptozotocin (STZ)-induced diabetic rat (27). Fermented *chaga* mushroom that contains polysaccharides, oligosaccharides, and polyphenolic compounds may very well be an ideal candidate to test for hypolipidemic and hepatoprotectic effects in a diabetic animal model. Little information is available, however, about the hypolipidemic and hepatoprotectic effects of the *chaga* mushroom in the Otsuka Long-Evans Tokushima fatty (OLETF) rat. The OLETF rat strain is one of the genetically manipulated animal models of spontaneous non-insulin-dependent diabetes mellitus (NIDDM) by possessing many similarities with human type 2 diabetes mellitus. Importantly, OLETF rats are characterized by the appearance of obesity and hypertriglycerolemia at about 6 weeks and NIDDM at about 30 weeks of age (28, 30). Thus, the OLETF rat is a preferred model of type 2 diabetes of the associated dyslipidemia (increased plasma triglycerides, cholesterol and free fatty acids) and obesity (abdominal fat weight) (29).

The primary objective of this study was to investigate the potential hypolipidemic and hepatoprotectic properties of fermented and unfermented *chaga* mushroom preparations in OLETF rats during the 26 to 34 weeks of age interval.

Materials and Methods

Total polyphenolic compounds of the *chaga* amushroom The *chaga* mushroom used in these studies was kindly donated by the Hong-Jae Green Co., Ltd. (Seoul, Korea). The dried fruiting bodies of *chaga* mushroom were cut into small pieces and then powdered in a blender. Fermented *chaga* mushroom was prepared according to the previously reported method (19). The polyphenolic compounds of *chaga* mushroom powder and fermented *chaga* mushroom were extracted by hot water for 3 hr. The polyphenolic compound concentrations were determined by the previously described method (31). Two mL of Folin-Ciocalteu's phenol reagent (Sigma Chemical Co., St. Louis, MO, USA) was added to 10 mL of water extracted chaga, mixed, and let stand for 5 min, followed by 2 mL of saturated sodium carbonate (Na_2Co_3) solution. The mixture was shaken and an aliquot taken for optical density measurement (640 nm) after 1 hr using a UV-VIS spectrophotometer (UV Mini 1240, Shimadzu, Japan). The polyphenolic compound concentrations were calculated from a standard curve of freshly prepared tannin solution.

Animal and experimental design The four-week old male OLETF and age-matched control LETO rats were obtained from the Tokushima Research Institute (Otsuka Pharmaceutical Experimental Co., Ltd., Tokushima, Japan), and housed individually in suspended wire-mesh stainless cages in a temperature (21-24°C) and light (08:00-20:00) controlled animal room. The composition of the semisynthetic diet is shown in Table 1. The LETO and OLETF rats were fed a standard nonpurified dry diet; tap water was available *ad libitum* until the age of 26 weeks. The OLETF rats were assigned to three groups (six animals per group): diabetic rats fed only the semisynthetic diet (OLETF control rats), the semisynthetic diet supplemented with *chaga* mushroom powder (OLETF CM-fed rats) and the semisynthetic diet supplemented with fermented *chaga* mushroom powder (OLETF FCM-fed rats). The mushroom supplementation in both the experimental groups was replaced with cellulose at a level of 5%(w/w).

Analytical procedures The end of the treatment period (20 to 22 weeks) included a final 12 hr of fasting. The rats were placed under light diethyl ether anesthesia and sacrificed by withdrawing blood from the abdominal aorta. The rat livers were quickly removed and weighed to the nearest gram. The serums were separated by centrifuging the bloods at 1,026×g for 15 min. The concentrations of serum total cholesterol and triglycerides, blood urea nitrogen and creatinine and the activities of AST and ALT were measured in the clinical laboratory of the Neodin Medical Institute (Seoul, Korea).

Histopathological examination Livers were carefully removed and small fragments for histomorphology were prepared for fixation with 4% paraformaldehyde prepared

Table 1. Dietary composition of the *chaga* mushroom experimental diets

Ingredients	LETO	OLETF		
		Control	CM	FCM
Casein	20	20	20	20
α-Corn starch	15	15	15	15
Corn oil	10	10	10	10
Cellulose	5	5	0	0
AIN-93 mineral mixture	4	4	4	4
AIN-93 vitamin mixture	1	1	1	1
L-Methionine	0.3	0.3	0.3	0.3
Sucrose	44.5	44.5	44.5	44.5
Choline bitartrate	0.2	0.2	0.2	0.2
CM[1)]	0	0	5	0
FCM[2)]	0	0	0	5

[1)]CM: *chaga* mushroom diet.
[2)]FCM: fermented *chaga* mushroom diet.

with 0.1 M phosphate buffered saline (PBS, pH 7.4), embedded in paraffin, and cut at approximately 6 μm for standard Hematoxylin & Eosin staining as described previously (32).

Statistical analysis The data from the experiments are presented as the mean ± SEM, and were analyzed using a one way analysis of variance (ANOVA), with the differences analyzed using the Duncan's new multiplerange test (33). A p value <0.05 was accepted as being a statistically significant difference.

Results and Discussion

Inonotus obliquus is a white rot fungus, a typical tree disease widely distributed over Europe, Asia, and North America that belongs to the Order Hymenochaetales, Family Hymenochaetaceae, and Class Basidiomycetes. This fungus is usually found as a hard sterile conk (sclerotia) termed '*Chaga*' on the *Betula* tree species (34). Since the 16th century, '*Chaga*' has been used as a folk remedy without apparent toxicity in Russia and other eastern European countries. A previous study indicated that the water-soluble polysaccharide extracted from *Inonotus obliquus* was a xylogalactoglucan composed of glucose, galactose, xylose, mannose, arabinose, and fructose (35). The yield of polysaccharides from *Inonotus obliquus* approximated 31% (11). We previously reported that the concentrations of crude polysaccharides extracted from dried *chaga* mushroom was 42.9% and from fermented *chaga* mushroom 39.1% (19); the other 3.8% of the fermented *chaga* mushroom was considered to be mostly oligosaccharides resulting from enzymatic hydrolysis of *Bacillus* sp. WRD-2 bacteria. Mushrooms with hypolipidemic properties, including that of the polysaccharides, were identified in such edible and medicinal mushrooms as *Garnoderma lucidum* (3), *Pleurotus ostreatus* (4), *Lentinus edodes* (5), *Flammulina velutipes* (5) and mixtures of these (6), and the *Inonotus obliquus* (13). We hypothesized that fermented *chaga* mushroom, containing both polysaccharides and oligosaccharides, may exert their hypolipidemic effects in the OLETF rat. Total polyphenolic compounds of *chaga* mushroom and fermented *chaga* mushroom were 0.8% and 0.9%, respectively.

As noted in Table 1, the body weights of the OLETF rat groups increased to a significantly greater extent than their age-matched LETO controls. There were no significant differences in body weight, however, among the three OLETF rat groups. The oral ingestion of the *chaga* mushrooms or fermented *chaga* mushrooms also caused no apparent changes in gross behavior or death. Thus, there were no harmful effects in the OLETF rats following oral administration of *chaga* mushrooms. The water consumption, however, was significantly higher in the OLETF control group than the LETO rats, but within the OLETF group the water consumption in the FCM-fed rats tended to be lower than the OLETF controls (Table 2). As has been reported previously, mushrooms administrated in the diet and drinking water of STZ diabetic rats and to genetically diabetic KK-Ay mice significantly reduced water consumption when compared to diabetic control animals (27, 36). In our previous experiment, we observed that the water intakes by STZ-induced diabetic rats were also significantly increased when compared with normal rats except for the animals fed a fermented mushroom yogurt (27). These results suggest that the reduction of water consumption associated with fermented *chaga* mushroom extracts in a diabetic state may be an important factor in improving the induced hyperglycemia of the diabetic animals.

A number of studies, the Framingham Study (37) and the WHO Multinational Study (38) have identified blood cholesterol level as an important indicator of cardiovascular risk in subjects with type 2 diabetes mellitus. In addition to cholesterol, particularly an increased triglyceride and the reduction of high-density lipoprotein (HDL)-cholesterol, are two factors closely associated with increased cardiovascular risk in the type 2 diabetic rats (39). The OLETF rat is an animal model of NIDDM characterized by mild obesity with visceral-fat accumulation and late-onset insulin resistance. This animal model customarily develops obesity and hypertriglycerolemia at about 6 weeks of age, insulin resistance at around 12 weeks, and

Table 2. Body weights and liver weights, food intake and water consumption in the LETO and OLETF rats

Ingredient	LETO	OLETF		
		Control	CM	FCM
Body weight				
Initial (g)	456.9±7.8[a]	664.4±14.6[b]	656.1±21.8[b]	655.8±34.0[b]
Final (g)	474.6±5.4[a]	680.5±3.9[b]	657.2±32.9[b]	682.5±34.4[b]
Food intake (g/day)	35.71±1.89[ns]	35.86±1.09	33.17±1.05	36.37±2.63
Water consumption (mL/day)	44.09±1.36[a]	79.58±11.92[b]	82.72±12.29[b]	66.43±17.03[b]
Liver weight (g)	12.41±0.31[a]	24.02±0.41[b]	23.41±0.96[b]	21.23±1.64[b]
Liver weight (%)	2.62±0.31[a]	3.53±0.08[b]	3.57±0.05[b]	3.10±0.14[c]

Values with different letters are significantly different at p<0.05.
Values are the means ± SE of six rats per study group.
ns: not significant.
CM: *chaga* mushroom diet.
FCM: fermented *chaga* mushroom diet.

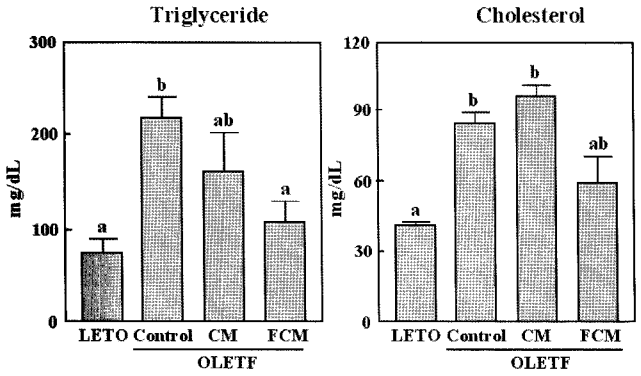

Fig. 1. Concentrations of triglyceride and total cholesterol in the LETO and OLETF rats. Values with different letters are significantly different at $p<0.05$. (mean±S.E., n=6). CM: *chaga* mushroom, FCM: fermented *chaga* mushroom.

NIDDM at around 30 weeks (28, 40). Plasma triglyceride and total cholesterol levels are in general strongly related to the degree of diabetes in the rats. Edible mushrooms then may be an ideal diet adjunct for the prevention of arteriosclerosis due to their ability to lower serum cholesterol and triglyceride concentrations. This effect may as well be caused due to, high fiber, lectins, glucans, glycoprotein, polysaccharide contents, and their physiological properties (1, 2, 41). Recently, hypolipidemic effects thought due to the consumption of mushrooms and their extracts were observed in STZ-induced diabetic, genetically diabetic and diet-induced hyperlipidemic rats (2, 6, 29). As shown by Fig. 1, serum triglyceride concentrations were significantly higher in the OLETF control than LETO rats in agreement with previous reports (40, 42). In the OLETF rat groups, the administration of fermented *chaga* mushroom significantly lowered the serum triglyceride concentrations in the OLETF control rats. The serum total cholesterol concentrations were also significantly higher in the OLETF control than in the LETO rats while in the OLETF group, the serum total cholesterol concentrations in the FCM-fed rats were slightly, but not significantly lower than in other two groups. Kim *et al.* (12) has reported that *Agaricus blazei* β-glucans and their oligosaccharides (derived from fermented hydrolyzing β-glucans with *Bacillus megaterium* glucanase) also significantly decreased the concentrations of serum triglycerides and total cholesterol in STZ-induced diabetic rats when compared with diabetic control rats and that this effect was more pronounced in oligosaccharides-feeding as opposed to β-glucans-feeding. Hypolipidemic active compounds include such polyphenolic substances as flavonoids, phenolic acid and tannin (43). In our study, the amount of polyphenolic compounds extracted from the powdered *chaga* mushroom and the fermented *chaga* mushroom approximated 0.8% and 0.9%, respectively. These results are consistent with that reported by Mau *et al.* (20) and Benoit *et al.* (44). Further, Lee and Jang (43) reported that total soluble polyphenolics in other mushrooms were in the order of *Agaricus bisporus* (0.88%), *Volvariella volvacea* (0.81%), *Flammulina velutipes* (0.78%), *Lentinus edodes* (0.72%), and *Pleurotus ostreatus* (0.59%). The *Inonotus obliquus* contains such polyphenolic compounds as hispolon and hispidin (25). We have hypothesized that the main efficacious ingredients of *Inonotus obliquus*, specifically the polysaccharidic and polyphenolic compounds may exert hypoglycemic and hypolipidemic effects when tested in experimental diabetic rats. The serum cholesterol concentrations were also significantly higher in the OLETF control than LETO rats. In the OLETF rat groups, the serum cholesterol concentrations in the OLETF FCM-fed rats were slightly lower than in other two groups. We conclude from our results that the fermented powder from fruiting bodies of the *chaga* mushroom lowers the elevated blood triglycerides and cholesterol concentrations in the obesity-related NIDDM of the OLETF rat.

The concentrations of serum creatinine and blood urea nitrogen (BUN) are shown in Fig. 3. The serum creatinine concentrations were significantly lower in the OLETF control than LETO rats. There were no significant differences in the serum creatinine concentrations among the OLETF rats groups, or the concentrations of BUN among all groups.

The activities of the hepatic AST and ALT enzymes in the rat are generally increased through metabolic changes induced by the administration of toxins e.g. STZ, alloxan, and the conditions of cirrhosis, hepatitis, and cancer (45). Thus, changes in serum AST and ALT activities can be used as markers for monitoring the extent of hepatic injury in diabetic animal models. There were no significant differences in the serum AST activities of the experimental groups,; however, ALT activities were significantly higher in the OLETF control than LETO rats (Fig. 2). In contrast, however, this increase in ALT activity was significantly lower in the CM and FCM-fed OLETF groups. The level of the serum ALT activity of the OLETF FCM-fed rats was comparable to the levels of the LETO rats. Jia *et al.* (46) has reported that serum AST activity has a tendency to decrease, whereas ALT activity tends to increase with age in OLETF rats. The activities of ALT and AST have previously been reported to be significantly higher in STZ-induced diabetic and genetically diabetic Zucker rats compared to their normal untreated and unmanipulated controls (2). These enzyme activities have also tended to be lower in the STZ diabetic and Zucker rats fed

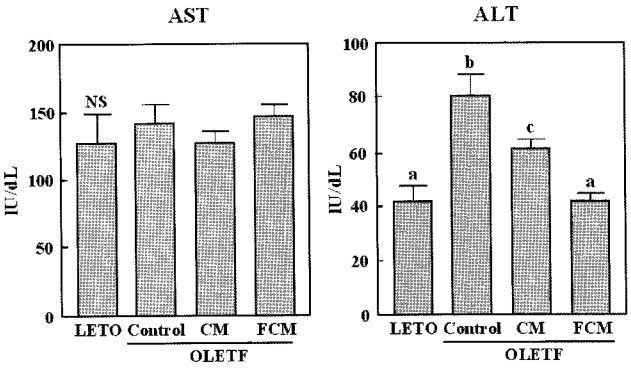

Fig. 2. Activities of serum ALT and AST in the LETO and OLETF rats. Values with different letters are significantly different at $p<0.05$. (mean±S.E., n=6). CM: *chaga* mushroom, FCM: fermented *chaga* mushroom.

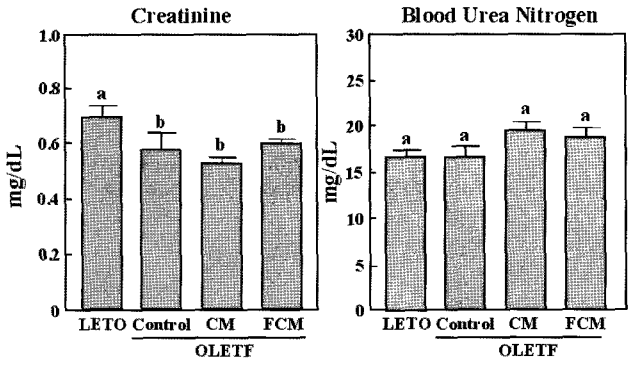

Fig. 3. Concentrations of creatinine and blood urea nitrogen in the LETO and OLETF rats. Values with different letters are significantly different at $p<0.05$. (mean±S.E., n=6). CM : *chaga* mushroom, FCM : fermented *chaga* mushroom.

fermented mushroom milk containing mushroom water extract (2). Yang *et al.* (47) reported that the AST and ALT activities were significantly reduced by *Lentinus edodes* exo-polymer (ED) in STZ diabetic rats.

Several mushrooms have been shown to have protective effects against chemically induced (D-galactosamine and carbon tetrachloride) hepatic injury (48). It is possible, therefore, that the reduction observed in the ALT activity following prolonged ingestion of fermented *chaga* mushroom is related to a hyperglycemic control mechanism in the OLETF rat. Figure 4 provides representative photomicrographs of sections of rat liver. The non-diabetic LETO rat liver, as expected, revealed no histopathology (Fig. 4-A), whereas those of the liver of the OLETF control rat indicates extensive fat deposition in the central zone of the hepatocytes (Fig. 4-B) as reported by others (46). The liver of the OLETF CM-fed rats appeared to have less fatty changes compared to OLETF controls (Fig. 4-C). Fat deposition in the hepatocytes was reduced while other histological observations were similar to those in the LETO group (Fig. 4-D). Crude extracts and polysaccharides isolated from such mushrooms as *Lentinus edodes*, *Tricholoma lobeyence*, and *Grifola frondosa* have also demonstrated a protective property against liver damage (47-49).

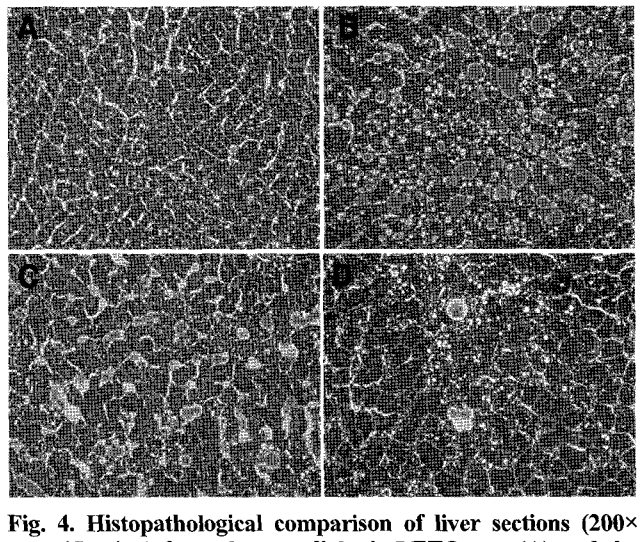

Fig. 4. Histopathological comparison of liver sections (200× magnification) from the non-diabetic LETO rats (A) and the diabetic OLETF rats (B, C and D). The liver sections were stained with haematoxylin and eosin (H & E) to demonstrate the general morphology. A: LETO, B: OLETF Control, C: OLETF CM (*chaga* mushroom), D: OLETF FCM (fermented *chaga* mushroom).

These results indicate that the ingestion of the fermented *chaga* mushroom may provide hypolipidemic and other beneficial effects in reducing the complications knowm to characterize obesity-related NIDDM in the OLETF rat and by extension perhaps in the human.

Acknowledgments

This study was supported by the Dong-A University Research Fund in 2004. The OLETF and LETO rats were donated by the Tokushima Research Institute, Otsuka Pharmaceuticals, Tokushima, Japan.

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