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Hypoglycemic Effects of Fermented *Chaga* Mushroom (*Inonotus obliquus*) in the Diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) Rat

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Abstract Changes in the levels of analytes in the blood and urine of a rodent animal model were taken as a measure of the hypoglycemic effects of a diet containing fermented *chaga* mushroom. These studies were conducted using the genetically manipulated diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rat. The effects of 8-week long diets that included either fermented (FCM) or non-fermented (CM) *chaga* mushroom powder (5% in the diet) on the OLETF rat were compared to the normal diet fed OLETF rat and the non-diabetic Long-Evans Tokushima Otsuka (LETO) rat. Hypoglycemia was tracked by measuring serum and urine concentrations of glucose, insulin, fructosamine, and leptin. Serum and urine levels of glucose, fructosamine, and leptin in the OLETF rats were higher than in LETO rats when fed normal diets but insulin levels did not differ between the two animal groups. The FCM rats were characterized by dramatically low levels of serum glucose and leptin in the OLETF rats whereas the levels of fructosamine and urine glucose trended lower in response to FCM. The serum leptin level in the CM-fed OLETF rat was also lower than that in the normal diet fed OLETF control. Serum concentrations of insulin in the OLETF rats were higher following FCM or CM feeding compared to the normal diet. These observations imply that (a) a dietary supplement of fermented *chaga* mushroom may contribute to a hypoglycemic effect in the OLETF rat, and (b) the increased blood insulin concentration following 8 weeks of an FCM diet may be important to the noted improvement in hyperglycemia.

Keywords: *chaga* mushroom (*Inonotus obliquus*), diabetes mellitus, OLETF rats, leptin

Introduction

Mushrooms have been used as healthy natural food substances as well as in traditional oriental-medicines throughout recorded history (1, 2). Many mushrooms are a source of physiologically active compounds that have been useful in efforts to develop natural medicines and pharmaceutical products (1-3). Interestingly, many species of mushrooms have also been reported to have hypoglycemic effects when studied in diabetic animal models (4-6). The biologically active components in mushrooms that are responsible for the hypoglycemic effects include inducible polysaccharides, polyphenolic compounds, peptidoglycans, terpenoids, lectins, and dietary fibers (5-9). Polysaccharides are perhaps the best known of the compounds as they possess the most potent hypoglycemic effects when introduced to animals made diabetic (5-9). Most findings of the hypoglycemic effects have been made in the streptozotocin (STZ)-, alloxan-treated, and genetically diabetic animal models that are treated with either whole mushroom powders (6, 10, 11) or water-soluble fractions of the mushrooms (5, 6). In addition to hypoglycemic properties, polysaccharides isolated from mushrooms have also been reported to have antitumor, antioxidant, and hypolipidemic attributes (12-14).

The fruiting bodies of *Inonotus obliquus*, commonly known as '*chaga* mushroom' have been used in traditional oriental medicines and for at-home treatments (14-16). The *chaga* mushroom is a typical tree fungus widely distributed through out Europe, Asia, and North America. The *chaga* mushroom has a history of use as a medical agent for the prevention and treatment of many human diseases, including various allergies and tumors, hypertension, and immunological disorders, presumably because of their polysaccharide, polyphenolic, and triterpene content (14-16).

Oligosaccharides produced by enzymatic hydrolysis from inulin, chicory, chitosan, and *Amorphophallus konjac*, have also been found to have hypoglycemic effects when administered to diabetic rats (17-19). The oligosaccharides consist primarily of di- and tri-saccharides, derived from the hydrolysis of β-glucans in the edible mushroom *Agaricus blazei* and hydrolyzed by the specific endo-β (1 → 6)-glucanase of *Bacillus megaterium* (20). These compounds have demonstrated anti-hyperglycemic, anti-hypercholesterolemic, and anti-arteriosclerotic activities in a diabetic rat model (20). We have previously determined that a water extract of the *chaga* mushroom, which was fermented by the *Bacillus* sp. WRD-2 strain, had cytotoxic effects on isolated cell lines (HCT-15 colon carcinoma and AGS gastric carcinoma) (21). Fermented *chaga* mushroom was also reported to have hypoglycemic effects in the type 1 STZ-induced diabetic rat (22). Fermented *chaga* mushroom containing polysaccharides and oligosaccharides may be a model candidate to test for hypoglycemic effects in diabetic animal models. However, a comparative study for possible hypoglycemic effects of whole *chaga* mushroom or fermented *chaga* mushroom in type 2 diabetic animal models may be necessary. The OLETF rat strain is a

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genetically manipulated animal model of spontaneous non-insulin-dependent diabetes mellitus (NIDDM) and an impaired glucose tolerance following intra-peritoneal glucose administration and possesses many similarities with human type 2 diabetes mellitus (23). The prime objective of this study was to investigate and to compare the effects of the fermented- and unfermented-fruiting body products of *chaga* mushroom (*I. obliquus*) on hypoglycemia in the diabetic OLETF rat model.

Materials and Methods

Crude polysaccharide preparation of *chaga* mushroom The dried fruiting bodies of *chaga* mushrooms were cut into small pieces and ground by a mechanical blender. The fermented *chaga* mushroom was subsequently prepared using the *Bacillus* sp. WRD-2 strain and cultured at 30°C on a rotary shaker for 2 days in a Luria-Bertani (LB) medium containing bactotryptone 10 g/L, yeast extract 5 g/L, and NaCl 5 g/L. The cultured strain solution was added to powdered *chaga* mushroom at a ratio of 1:10 (v/w) and incubated at 30°C for 30 days and dried in a 60-70°C oven. The crude polysaccharides from water-extracts of both fermented and non-fermented *chaga* mushrooms were prepared as described previously (5, 24). Five volumes ethanol (v/w) were added to the mushroom extracts to precipitate the polysaccharides (crude fractionation). The precipitate was collected, washed with ethanol and the concentration of the water-soluble polysaccharides determined by the phenol sulfuric acid method. The oligosaccharides obtained from water-extracts of both fermented and non-fermented *chaga* mushrooms were identified by thin layer chromatography (TLC) on silica gel-precoated plates (Kiesel gel $60F_{254}$, Merck, Germany). The products were developed by means of multiple ascents using a mixed solvent system of 1-butanol/ethanol/water (5:5:3, v/v), and detected by spraying with 3% sulfuric acid in methanol and heat (140°C for 10 min).

Animals and experimental design Four weeks old male OLETF (N=18) and LETO (N=6) rats were obtained from the Tokushima Research Institute (Otsuka Pharmaceutical Experimental Co., Ltd., Tokushima, Japan). The animals were housed individually in suspended wire-mesh stainless steel cages at room temperature (21-24°C) and with lights on between 08:00 and 20:00 hr. The composition of the semisynthetic diet was as follow (g/kg); starch 400, casein 200, sucrose 200, corn oil 100, cellulose 50, mineral mixture (AIN 93 G-MX mineral mix; MP Biomedicals, Germany) 35, vitamin mixture (AIN 93 VX vitamin mix; MP Biomedicals) 10, DL-methionine 3, and choline bitartrate 2. All animals were fed a standard non-purified diet with tap water provided *ad libitum* until the age of 26 weeks. The OLETF rats were assigned to three groups of six rats each as follows: diabetic rats fed the semisynthetic diet (OLETF control rats), the semisynthetic diet supplemented with *chaga* mushroom powder (OLETF CM rats), and the semisynthetic diet supplemented with fermented *chaga* mushroom powder (OLETF FCM rats). The study period was of 8 weeks duration (from 26 to 34 weeks of age). Mushroom supplementation in both the OLETF CM or FCM rats was replaced with cellulose at a level of 5%(w/w).

Analytical procedures At the end of the 8-week treatment period and after 12 hr of fasting, the rats were lightly anesthetized with diethyl ether and a blood sample withdrawn from the abdominal aorta. Serum was obtained following centrifugation of blood samples at 1,026×g for 15 min. The serum insulin concentration was measured using an immunoradiometric assay kit (Biosource; Europe S.A., Nivelles, Belgium) as was the concentration of serum leptin (ELISA Rat Leptin Kit; Linco, St. Charles, MO, USA). Serum concentrations of fructosamine were measured by an enzymatic method using a kit (GlyPro; Genzyme, Cambridge, MA, USA). Urine samples were collected from the base of the metabolic cages over the final two days of the experimental period and urinary glucose concentrations were measured in a Fuji DRI-Chemiclinical Chemistry analyzer (DRI-CHEM 3500; FUJI, Tokyo, Japan).

Oral glucose tolerance test (OGTT) The OGTT was performed after an overnight fast (water allowed *ad libitum*) at the midpoint of the study (4 weeks). The blood glucose concentrations were measured using a Lifescan glucose meter and One Touch test strips (Lifescan Inc., Milpitas, CA, USA), with whole blood samples collected from the tail vein before and at 30, 60, 90, 120, and 150 min after the oral administration of the glucose solution (1.0 g/kg bw).

Statistical analyses The data are presented as the mean± SEM, and were statistically analyzed using one way analysis of variance (ANOVA), with the differences further analyzed using Duncan's new multiple-range test (25). A p value <0.05 was accepted as being significantly different.

Results and Discussion

Many biologically active forms of beta-glucans, which are isolated from mushrooms and having (1→3) - and (1→6)-linkages, are distributed in soluble and insoluble dietary fractions and have been widely used in medicine for their anti-tumor and anti-diabetes properties (26). Mizuno *et al.* (14) and Lee and Kim (26) reported that the extractable yield of crude polysaccharides and the total glucans (α- and β-glucans) isolated from *chaga* mushroom are 31.0 and 5.23%, respectively. The yield of crude polysaccharides from non-fermented and fermented *chaga* mushrooms were 42.9 and 39.1%, respectively (Table 1), with 3.8% of the fermented *chaga* mushroom considered to be mostly oligosaccharides due to enzymatic hydrolysis by the *Bacillus* sp. WRD-2 strain and which were subsequently identified as the di- and tri-saccharides (Fig.

Table 1. Percentage of polysaccharides and oligosaccharides in non-fermented and fermented *chaga* mushrooms

Mushroom preparation	Polysaccharide (%)	Oligosaccharides (%)
Non-fermented *chaga*	42.9	0
Fermented *chaga*	39.1	3.8

1). Kim *et al.* (20) reported that the oligosaccharides, derived from enzymic hydrolysis of the β-glucans (molecular weight 30-40 kDa) of *A. blazei* Murill with an endo-β-(1→6)-glucanase from *B. megaterium* were mainly di- and tri- oligosaccharides and that these oligo-saccharides had approximately twice the activity of the parent β-glucans with respect to a hypoglycemic effect in a rat model. Oligosaccharides produced by the enzymatic hydrolysis of inulin, chicory, and *A. konjac*, were also reported to have hypoglycemic effects in diabetic rats (17-19). Further, hypoglycemic effects have also been attributed to edible and medicinal mushrooms such as, including those polysaccharides, *Ganoderma lucidum* (6, 10), *Auricularia auricula judae* Quel. (5), *Tremella aurantina* (8), *Grifola frondosa* (27), and *I. obliquus* (14). We had hypothesized that the FCM, containing polysaccharides and oligosaccharides, may exert a more pronounced hypoglycemic effect in the OLETF rat when compared with the CM containing only polysaccharides.

The body weight of rats in the OLETF groups were increased significantly compared to age-matched LETO rats (Fig. 2), but there was no significant difference in body weights between the OLETF rat groups and thus no dietary effect of CM or FCM on body weight can be stated. A survey of the literature also failed no reveal any reports of changes in body weights due to diets supplemented with water soluble polysaccharides or other whole mushroom powders such as oyster (*Pleurotus ostreatus*), maitake (*G. frondosa*), shiitake (*Letinus edodes*), or enokitake (*Flammulina velutipes*) in diabetic mice (5), Wister (28), and F344/DuCrj rats (29). The oral ingestion of the CM or FCM mushroom extracts caused no apparent

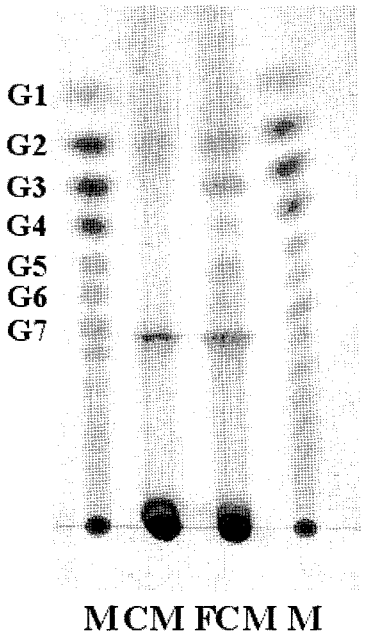

Fig. 1. Thin-layer chromatography of the water extracts from non-fermented and fermented *chaga* mushrooms. Standards used are malto-oligosaccharides (M) containing G1 (glucose), G2 (maltose), G3 (maltotriose), G4 (maltotetraose), G5 (maltopentaose), G6 (maltohexaose), and G7 (maltoheptaose). CM, non-fermented *chaga* mushroom; FCM, fermented *chaga* mushroom.

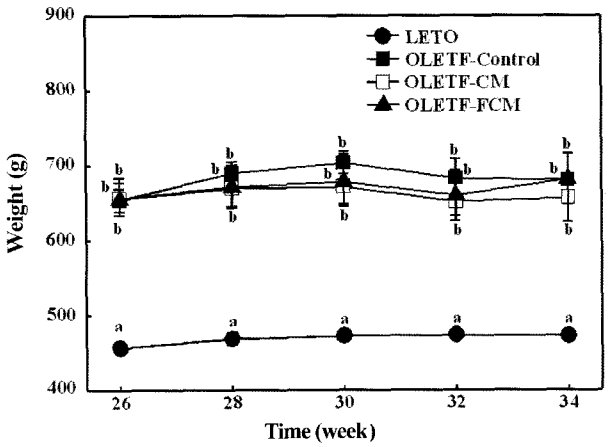

Fig. 2. Body weight changes in LETO and OLETF rats. Values with different letters represent a statistical difference at $p<0.05$. (mean±SE, n=6). CM, non-fermented *chaga* mushroom; FCM, fermented *chaga* mushroom.

behavioral changes or animal deaths. Thus, there were no obvious harmful effects observed in the OLETF rats as a result of the oral administration of *chaga* mushrooms.

The OLETF rat is an animal model of NIDDM characterized by mild obesity with visceral-fat accumulation and late-onset insulin resistance. The OLETF rat develops its obesity and hypertriglyceridemia around week 6 of age, insulin resistance by 12 weeks, and NIDDM at 30 weeks (23, 30, 31). It is well known that plasma triglyceride levels are a strong indication of the degree of diabetes in the rat (11, 22, 30). In our preliminary study, a decreased plasma triglyceride level in the STZ-diabetic rat fed an FCM-diet related to the degree of diabetic control (22), but this was not observed in the present animal fed non-fermented *chaga* mushroom study. The present study has demonstrated an association between plasma triglycerides and glucose levels in the OLETF rats fed the FCM diet. The concentrations of blood glucose in the non-fasting OLETF control rats gradually increased over time as the animals continued their body weight increment. The glucose levels at the end of the 8 weeks of study (34 weeks old) in the OLETF control rat was significantly higher than that of the LETO rats (Fig. 3), and in agreement with previous reports (32, 33). In comparison to the OLETF CM rat, the increased level of glucose in the OLETF control rat at a comparable 8 weeks of the experiment was slightly higher numerically but not significant statistically.

The blood glucose concentration in the OLETF FCM rat, however, was drastically decreased by the 8-week feeding period (Fig. 3). Previous studies have shown that blood glucose concentrations were significantly decreased in genetically diabetic mice and in the STZ- and alloxan-induced diabetic mice, fed diets containing water-soluble polysaccharide or powdered mushrooms such as *A. blazei*, *A. auricular*, *G. lucidum*, and *T.aurantia*, (5, 6, 20). These results, therefore, suggest that the intake of a mushroom component(s) might regulate blood glucose levels in both the genetically and chemically induced diabetic animal models.

The changes in concentration of blood glucose at

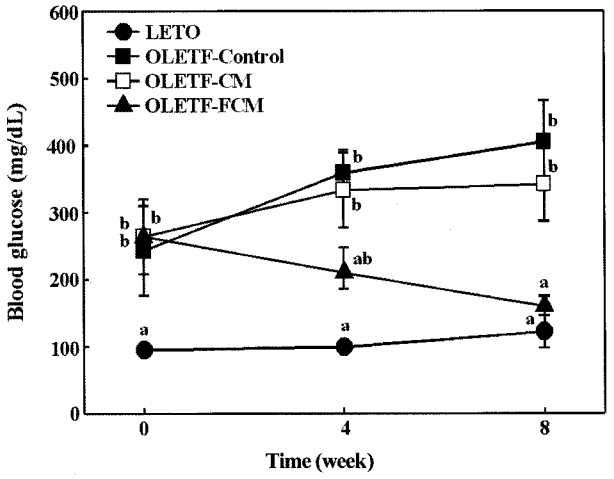

Fig. 3. Blood glucose concentrations in the non-fasting LETO and OLETF rats. Values with different letters represent statistically significant differences at $p<0.05$. (mean±SE, n=6). CM, non-fermented *chaga* mushroom; FCM, fermented *chaga* mushroom.

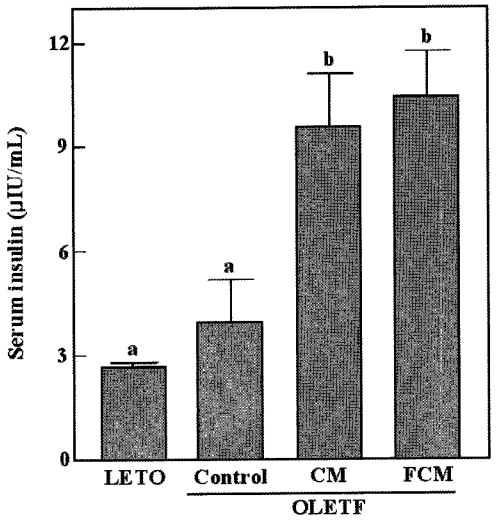

Fig. 5. Serum insulin concentration in LETO and OLETF rats. Values with different letters are significantly different at $p<0.05$. (mean±SE, n=6). CM, non-fermented *chaga* mushroom; FCM, fermented *chaga* mushroom.

various times in response to oral glucose administration are shown in Fig. 4. The OGTT was conducted in the overnight fasted rat at 4 weeks following initiation of their experimental diets. Although the basal blood glucose concentrations for the LETO and OLETF rats were nearly similar before oral glucose administration, the level was significantly decreased in the LETO rat compared to OLETF control rat after glucose loading. The blood glucose concentrations in the OLETF rat groups were barely influenced by oral glucose administration. The blood glucose concentration of the OLETF rats fed diets with CM or FCM showed a trend to decrease at 120 and 150 min after glucose administration in comparison to the OLETF normal diet controls. Yuan and their colleagues (5) reported that polysaccharides fed to genetically diabetic KK-Ay mice resulted in an improved tolerance to an intraperitoneal glucose load. The oligosaccharides produced by enzymatic hydrolysis of inulin, chicory, chitosan, *A. konjac* and the medicinal mushroom *A. blazei* Murill also reported to have hypoglycemic effects in diabetic rats (17-20). Chai and Rhee (34) have reported that 10% xylooligosaccharides improves oral glucose tolerance and hyperglycemia in the STZ-diabetic rat.

Recent evidence indicates that fructosamine can be used as a clinical tool to assess long-term blood glycemic control (i.e., within 1-3 weeks) in the human diabetic patient (35). Thus, it is of interest that OLETF rats fed fermented *chaga* mushroom in their diet improved their long-term glycemia responses as indicated by a significant decrease in serum fructosamine concentrations (Fig. 6). Ingestion of fruiting bodies and the acidic polysaccharide

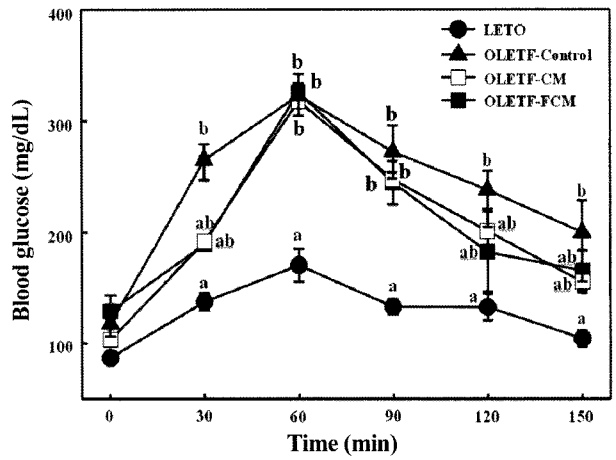

Fig. 4. Blood glucose changes in the oral glucose tolerance test (OGTT) in LETO and OLETF rats. Values with different letters represent statistical differences at $p<0.05$. (mean±SE, n=6). CM, non-fermented *chaga* mushroom; FCM, fermented *chaga* mushroom.

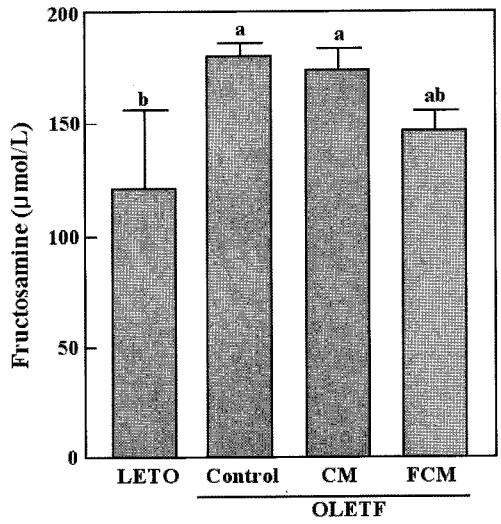

Fig. 6. Serum fructosamine concentration in LETO and OLETF rats. Values with different letters are significantly different at $p<0.05$. (mean±SE, n=6). CM, non-fermented *chaga* mushroom; FCM, fermented *chaga* mushroom.

glucuronoxylomannan of *Tremella mesenterica* significantly reversed increased fructosamine levels in the STZ-induced diabetic rat thereby suggesting that fruiting bodies and polysaccharides of *T. mesenterica* have long-term anti-hyperglycemic effects (36). Therefore, the *chaga* and *Tremella* mushrooms may be considered as potential anti-hyperglycemic foods or nutraceuticals useful in the future treatment of diabetes.

To our knowledge, this is the first study to examine the effects of fermented and non-fermented *chaga* mushroom on serum leptin concentrations in the OLETF rat. Leptin is generally produced by adipose tissue and acts as a saity feedback signal to the hypothalamus for energy homeostasis by reducing food consumption, increasing energy expenditure or effecting a combination of the two processes. Because serum leptin levels are correlated with body fat mass, leptin levels can be used as an index to predict obesity-related diseases (37, 38). Circulating leptin levels in the OLETF rat were increased with age to be 2-3 times higher than in the lean control LETO rat. Thus, the OLETF rat is hyperleptinemic by 8 weeks of age as previously reported (39). The present study observed that serum leptin levels in the OLETF control rats were 3.9-fold higher than in the LETO rats at 34 weeks of age (Fig. 7).

The elevated serum leptin in the OLETF rat, however, was significantly reduced by both CM and FCM (Fig. 7). Several hypoglycemic components have been shown to prevent hyperglycemia in the OLETF rats that accompanied with a significant reduction of serum leptin concentration (38). Therefore, some as yet unidentified component of the fermented *chaga* mushroom might improve leptin resistance in the OLETF rat which might contribute to an improved glycemic status in this model, although the precise mechanism is not known.

The urinary glucose concentration was significantly high at end of the 8-week feeding period in the OLETF control rats compared to the LETO animals (Fig. 8). In the FCM fed OLETF rat group, a significant decrease in urinary glucose concentration was noted when compared

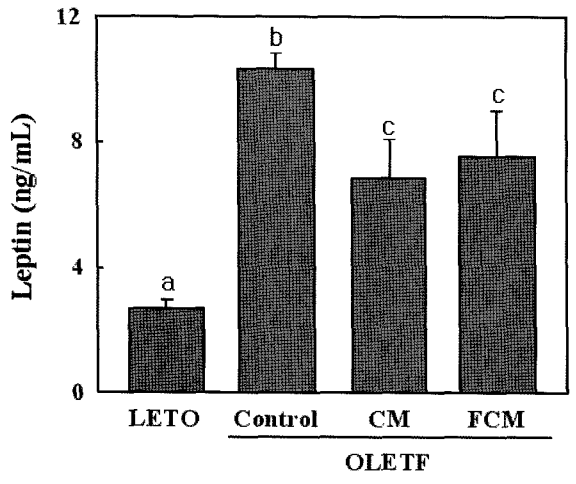

Fig. 7. Serum leptin concentration in LETO and OLETF rats. Values with different letters are significantly different at $p<0.05$. (mean±SE, n=6). CM, non-fermented *chaga* mushroom; FCM, fermented *chaga* mushroom.

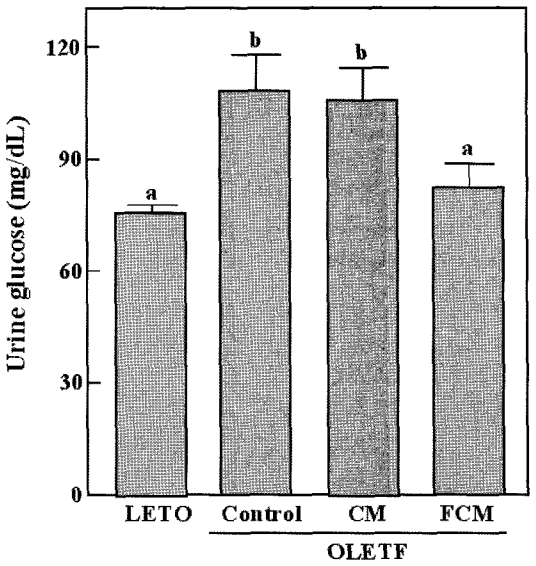

Fig. 8. Urinary glucose concentration in LETO and OLETF rats. Values with different letters are significantly different at $p<0.05$. (mean±SE, n=6). CM, non-fermented *chaga* mushroom; FCM, fermented *chaga* mushroom.

to the corresponding diabetic control rats (OLETF normal diet) suggesting that this effect was dependent on the degree of blood glucose control due to the ingested fermented *chaga* mushroom diet. A previous study in the KK-Ay mouse also indicated that mushroom polysaccharides reduced urinary glucose concentrations significantly (5). The pathophysiological characteristics involving reduced insulin secretion and increased insulin resistance have been investigated previously in the OLETF rat as a model of human type-2 diabetes mellitus (32, 33).

The inhibition of insulin secretion via β-cell destruc-tion in the pancreatic islets of the OLETF rat model has been closely correlated with elevated blood glucose levels. Previous studies in the OLETF rat showed that blood insulin levels gradually increase as the animal ages (until 30 weeks) and then decreases in parallel with the onset, progression, and maintenance of a hyperglycemic state (32, 33). Studies with the BRIN-BDll pancreatic β-cell line or isolated islet cells of rats fed mycelial *L. edodes* showed that an aqueous extract of the mushroom stimulated insulin secretion (40, 41). The blood insulin concentration in the OLETF rat group, in the present study, was dramatically increased following the administration of CM or FCM when compared with the response in diabetic OLETF control rats (Fig. 5). How-ever, in our previous study using the STZ-diabetic rat, the FCM diet but not the CM diet significantly suppressed development of hyperglycemia by increasing plasma insulin levels (22). Thus, these results enable the speculation that FCM is likely more effective in both type 1 and type 2 diabetic animal models than is the CM diet when consumed at comparable dietary weight percentages. Polysaccharides isolated from the fruiting bodies of *G. lucidum* and *Phellinus baumii* reportedly stimulate insulin secretion from pancreatic islet cells (*in vivo* and *in vitro)* thereby protecting against alloxan- and STZ-induced pancreatic

islet cell damage and that this protection in turn promotes insulin synthesis which works to lower blood glucose concentrations (38, 42). The polysaccharides from *G lucidum* also increased blood insulin concentrations in the normal mouse and rat (10, 43). Hu *et al.* (44) has reported that water-soluble polysaccharides from the fermented broth of the edible mushroom, *Pleurotus citrinopileatus,* alleviated hyper-glycemia in STZ-induced diabetic rats, also by protecting the damaged pancreatic cells. These results suggest possible hypoglycemic mechanisms that are under physiological glucose control and permit stimulatory effects of polysaccharides on insulin, which depends mainly on the extracellular Ca^{2+} influx to the pancreatic beta-cell (10). Significantly improved STZ-induced hyperglycemia as a result of the administration of calcium or zinc has been reported (45, 46) and was key to the stimulation of insulin secretion in pancreatic β-cells related to endogenous calcium levels. An increase of calcium and zinc in the pancreas of the Zucker fatty-diabetic rat fed a fermented mushroom milk product containing a water-extract of mushroom, in our preliminary study, might then correlate to the degree of diabetic control. Thus, these observations lead to the speculation that the hypoglycemic effects of FMC and MC supplemented diets in the OLETF rat, may be partially due to enhancement of insulin synthesis and secretion from the pancreas. Indeed, a water-extract of the *maitake* mushroom that contained β-glucans positively influenced glucose and insulin metabolism by enhancing peripheral insulin sensitivity in the insulin-resistant KK mouse (4).

We conclude that the results of our studies in the OLETF diabetic rat model supports the notion that fermented *chaga* mushroom included in a normal diet might have favorable hypoglycemic effects on obesity-related type 2 diabetes.

Acknowledgments

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