Chinese Journal of Physiology 57(4): 198-208, 2014
DOI: 10.4077/CJP.2014.BAC245

Effect of *Pleurotus tuber-regium* Polysaccharides Supplementation on the Progression of Diabetes Complications in Obese-Diabetic Rats

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Abstract

In this study, the effect of mushroom extracellular polysaccharides on fatty acid composition and liver peroxisome proliferator-activated receptor-alpha (PPAR-α) expression in obese-diabetic rats was investigated, and distinguished the association among anti-obesity, hypoglycemic and hypolipidemic properties. Extracellular polysaccharides from three different strains of *Pleurotus tuber-regium* were extracted and labeled as HP (high-percentage), MP (medium-percentage) and LP (low-percentage). Obese-diabetes (OD) was induced by chronic high-fat diet plus streptozotocin (STZ) injections. Simultaneously to the diet, polysaccharides were orally administered to OD groups (20 mg/kg body weight/8-week), and categorized into OD+HP, OD+MP and OD+LP groups (n = 10/group), respectively. High-fat diet plus STZ-induced hyperglycemia was prominently attenuated by polysaccharides. Increased fatty acid component n-6/n-3 ratio in liver and plasma of obese-diabetic rats was attenuated, while, reduced MUFA/PUFA and MUFA/SFA ratios were restored ($P < 0.01$) with polysaccharides treatment. Furthermore, elevated serum total cholesterol, triglycerides and low-density lipoprotein (LDL) concentrations were controlled, and parallel restoration of decreased high-density lipoprotein (HDL) levels were found with polysaccharides supplementation. This hypolipidemic property might be associated with up-regulated liver PPAR-α mRNA expression and protein levels ($P < 0.01$). These findings concluded that stable fatty acid components and activated PPAR-α by polysaccharides may contribute to its hypoglycemic and hypolipidemic properties. Therefore, *P. tuber-regium* could be considered as nutritional supplement to treat diabetic complications.

Key Words: dyslipidemia, fatty acid composition, hyperglycemia, mushroom, obesity

Introduction

Nowadays changes in dietary habits have become a serious health concern around the world (5). Consumption of chronic high-fat diet has been shown to affect the membrane fatty acid composition that

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Received: September 30, 2013; Revised (Final Version): November 2, 2013; Accepted: November 13, 2013.
 ISSN : 0304-4920. http://www.cps.org.tw

leads to diminish the metabolic rate, thereby increase susceptibility to obesity (35). In Western countries, above 50% of adult population aged between 35 and 65 years were either overweight or obese. Obesity is closely associated metabolic syndrome, including type 2 diabetes and cardiovascular disease (CVD) (28, 42). Latest International Diabetes Federation (IDF, 2012) calculations revealed that the number of people living with diabetes is expected to rise from 366 million in 2011 to 552 million by 2030. Currently, a large number of population with diabetes are from China, India and USA (39), which might be due to the drastic change in dietary habits and lifestyle.

Diabetes is not only a metabolic disease characterized by hyperglycemia, but also represents dyslipidemia, an independent risk factor for CVD (30, 33). Studies demonstrated that alterations in membrane fatty acid composition (29) and peroxisome proliferator-activated receptor-α (23) under hyperglycemia/diabetic condition may further exacerbate the disease. Although some oral hypoglycemic agents and insulin are partially helping to manage the diabetes, these drugs are less effective in normalizing the lipid levels, conversely produce additional burdens to diabetic patients (16). The hypoglycemic and hypocholesterolemic properties of some edible mushroom are experimentally demonstrated in diabetes rats without adverse effects (24, 41). Mushrooms are nutritionally important and included as a major constituent in the regular diet among East Asian counties. *Pleurotus tuber-regium*, known as 'king tuber mushroom', is an edible mushroom that has been used in Chinese medicine to treat several human ailments (4, 20, 31). Fruiting bodies of *P. tuber-regium* are rich in protein, while sclerotium is rich in fibers, especially, rich non-starch polysaccharides (25). These polysaccharides are mainly composed of bioactive β-glucans, which are responsible for its pharmacological actions, including antidiabetic properties and modulation of hematopoiesis (7, 19). Since mushroom polysaccharides possess diverse biological activities, *P. tuber-regium* with abundant polysaccharides considered as important medicinal mushroom that could be used to treat metabolic syndrome. However, mushroom cultivation by traditional methods is a time taking process, and extracted polysaccharides may exhibits divergent results due to the variance in bioactive compounds. This ambiguous can be overcome by applying the submerged culture method to get bioactive compounds in a short-period under controlled environment (26).

Hence, in this study we adopted submerged culture method, and extracellular polysaccharides were extracted from the culture media of three different strains of *P. tube-regium*. In view of its potential therapeutic effects, we assume that chronic polysaccharides administration may delay/prevent the progression of diabetes and associated adverse effects. Therefore, we investigated the influence of polysaccharides supplementation on altered fatty acid profile and PPAR-α expression in experimental obese-diabetic rats. Furthermore, antihyperglycemic and antilipidemic properties of polysaccharides were examined, and attempted to distinguish the association among anti-obesity, antihyperglycemic and antilipidemic properties.

Table 1. Components in standard diet and high-fat diet

Components	Standard Diet (g/100 g)	High-Fat Diet (g/100 g)
Corn Starch	46.57[a]	46.57
Dextrin	15.5	15.5
Sucrose	10	10
Casein-Vitamin Free	14	14
Powdered Cellulose	5	5
Soybean Oil	4	4
AIN-93 Mineral Mix	3.5	3.5%
AIN-93 Vitamin Mix	1	1%
Choline Bitartrate	0.25	0.25%
L-Cystine	0.18	0.18%
t-Butylhydroquinone	0.0008	0.0008%
lard	0	18
Calorie (Kcal/30 g/day)	114	141

[a]Individual components are expressed in percentage.

Materials and Methods

Chemicals

Streptozotocin (STZ) and other chemical used in this study were obtained from the Sigma Chemical Co., (St Louis, MO, USA). Mushroom culture media were obtained from the Mycotheque catholique de Louvain, Louvain-la-Neuve, Belgium.

Animal Maintenance and Diet

Wistar strain male albino rats (n = 50) aged 2-month (weighing 230 ± 20 g) were obtained from the National Laboratory of Animal Breeding and Research Center, Taipei, Taiwan. All rats were maintained under hygienic conditions with controlled temperature (23 ± 2°C) and alternating 12-h dark and light cycle. After acclimatization to laboratory conditions, except control, all rats had free access to high-fat diet (22%) and water. The standard diet AIN-93M formula (4% fat) was modified by adding additional lard (18%) to the fat portion in order to get the high-fat diet (22%, Table 1). All experiments were conducted according to the ethical guidelines and this study was approved

by the Institutional Animal Ethics Committee of Shih-Chien University.

Preparation of Extracellular Polysaccharides and Estimation of Glucans

Polysaccharides were extracted from three different submerged culture media of *Pleurotus tuber-regium* strains, such as MUCL-39359, MUCL-44597 and MUCL-44822. The culture media were obtained from the Mycothèque catholique de Louvain, Louvain-la-Neuve, Belgium. Polysaccharides extraction was carried-out at the Institute of Biochemistry and Molecular Biology, National Yang-Ming University, Taipei, as described by Dong *et al*. (10) and Yoshioka *et al*. (43). Briefly, the mycelia of *P. tuber-regium* were cultured in 300 ml Erlenmeyer flasks containing 100 ml broth for 20 days (6.5% glucose, 0.30% soy peptone, 1% yeast extract, 0.01% $MgSO_4$ and 0.02% KH_2PO_4 in distilled water at pH 5.5). The culture broth was separated from the mycelia by filtration, and then freeze-dried for experimental use. The amount of extracellular polysaccharides from each strain was quantified by Phenol-Sulfuric acid method. Glucans were screened in each strain by the Mushroom and Yeast β-glucan assay kit provided by Megazyme International Ireland Ltd., (Bray, Ireland). The values were expressed in percentage (%) per total dry weight of mushroom.

Freeze-dried polysaccharides were freshly prepared each time prior to administration by dissolving in required quantity of HPLC grade water. Based on the percentage of polysaccharides presence in each strain, we labeled MUCL-39359 as HP (high percentage), MUCL-44597 as MP (medium percentage) and MUCL-44822 as LP (low percentage) polysaccharides.

Induction of Obesity and Diabetes (Hyperglycemia)

From the first day of experiment, rats had free access to high-fat diet to induce obesity, and chronic low dose of STZ was injected to produce hyperglycemia. The combined actions of high-fat diet plus STZ injections throughout the study projected to increase the obesity levels, and simultaneously maintain the hyperglycemia. This scenario leads to the progression of obese-diabetes condition in rats over the period of 8 weeks.

Prior to STZ injection all rats were fasted for 12 h, and freshly prepared STZ (0.1 M citrate buffer, pH 4.5) was injected intraperitoneally (10 mg/kg body weight) on every other day in a total volume of 1 ml along with nicotinamide (30 mg/kg b.w.). Nicotinamide was combindly administered with low dose of STZ to induce a rat model with postprandial hyperglycemia, which mimics the features of type 2 diabetes (35). At the tested concentration, neither death nor any other adverse effects were observed throughout the study. Fasting blood glucose levels were monitored for every three days, and rats with hyperglycemia (≥200 mg/dl, after 6 week) and increased whole body weight were confirmed as obese-diabetic rats.

Experimental Design and Treatment

Fifty rats were divided into five groups, ten rats in each. First group served as healthy control, and remaining four groups were fed a high-fat diet and injected STZ to consider as experimental obese-diabetes (OD) groups. Three OD groups were supplemented with extracellular polysaccharides extracted from three different strains of mushroom along with high-fat diet and STZ injections. The detailed treatments were as follows:

Group I. Control (CON): Rats (n = 10) in this group relied on regular standard diet (AIN93M) throughout the study, and served as healthy normal control group for better comparison with obese-diabetes and treated groups. Control rats were administered orally with 0.9% saline for equivalent handling with treatment groups.

Group II. Obese-diabetes (OD): All rats (n = 10) in this group served as OD control without any treatment, however, received low dose of STZ injections and fed a high-fat diet as described in the section 'induction of obesity and diabetes'. Saline (0.9%) was administered orally for equivalent handling with treatment groups.

Group III. OD plus high-percentage polysaccharides (OD + HP): Polysaccharides extracted from *P. tuber-regium* strain MUCL-39359 that contains high-percentage of polysaccharides (HP) were orally supplemented to ten rats (20 mg/kg b.w./day for 8 week, gavage).

Group IV. OD plus medium-percentage polysaccharides (OD + MP): Rats were administered with medium-percentage of polysaccharides (MP) extracted from MUCL-44597 strain of *P. tuber-regium* at the dose of 20 mg/kg b.w/day for 8 weeks by oral gavage.

Group V. OD plus low-percentage polysaccharides (OD + LP): This group of rats received the same dose (20 mg/kg b.w./day, gavage) of low-percentage polysaccharides (LP) derived from *P. tuber-regium* strain MUCL-44822 for the same period.

Blood samples were collected from all the groups under fasting condition between 8.00 and 9.00 am, for every three days. After completion of 8-week treatment, liver tissue was isolated. Simultaneously blood samples were also collected and separated plasma for assays. Body weights on every other day, and energy intake on every day were recorded throughout the study. The average daily energy intake (Kcal

per gram diet) was calculated by: average food intake per day (g) / provided food (30 g) × number of calories present in 30 g. The number of calories in standard (control) diet is 114 Kcal, while high-fat diet contained 141 Kcal.

Monitoring of Fasting Blood Glucose, Serum Insulin and Adiponectin Concentrations

Fasting blood samples were collected from the tail vein, and immediately determined the blood glucose levels by the glucose analyzer (Lifescan, Milpitas, CA, USA). For insulin levels, about 200 µl blood sample was transferred into labeled centrifuge tubes and then centrifuged at 3,500 rpm for 10 min to obtain serum. As described in the protocol, serum insulin levels were estimated by using commercial ELISA kits (Diagnostic Systems Laboratories, Webster, TX, USA). The serum sample was quantified on ELISA analyzer (Tecan Genios, A-5082, Austria).

Adiponectin, known as adipocyte complement-related protein of 30 kDa (ACRP30) was estimated in serum of rats by AssayMax Rat Adiponectin ELISA Kit provided by Assaypro LLC & Angiopharm LLC (St. Charles, MO, USA).

Measurement of Serum Lipid Profiles

Since obesity is strongly associated with profound changes in lipid metabolism, serum total cholesterol (TC), triacylglycerol (TG), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) levels were monitored from the fresh samples. These estimations were done spectrophotometrically using Vitros DT60 II analyzer (Johnson and Johnson Clinical Diagnostics Inc., Rochester, NY, USA). All the values were expressed as mg/dl.

Analysis of Plasma and Hepatic Fatty Acid Composition by Gas Chromatography

The phospholipids extracted from samples were dissolved in 1 ml of 14% boron trifluoride methanol (BF3-methanol, Sigma®), and 100 ml of heptadecanoic acid (Margaric acid, C17:0, Sigma®) was added as internal standard, then methylated reaction was performed by water bath heating at 100°C for 30 min. Finally, 2 ml pentane and 1 ml H_2O were added and centrifuged for 10 min; collected supernatant was re-dissolved in 100 ml n-hexane then injected into Gas Chromatography (Trace GC, Thermo Finnigan) for fatty acid profile analysis. The Gas Chromatography was equipped with a 30 m, 0.32 mm ID capillary column (cross-linked polyethylene glycol-TPA phase, Sulpelco®) and frame ionized detector. The injector and detector temperatures were 230°C and 270°C respectively, and the split ratio was 100:1. Oven temperature was set at 160°C for 4 min at the initial stage, and was increased by 2.5°C/min to 225°C then kept for 20 min. Peaks were recorded and interpreted by a programmable integrator (Chromcard for Trace). Fatty acid profiles in plasma and liver samples were identified according to the retention time of appropriate standard fatty acid methyl esters.

RT-PCR for PPAR-α mRNA Expression

Total RNA from the liver samples was extracted using Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA), followed by RT-PCR. PPAR-α upstream primer sequence was 5'-TGAACAAAGAC-GGGATG-3', and downstream primers sequence was 5'-TCAAACTTGGGTTCCATGAT-3'. The length was within 106 bp. Similarly, β-actin upstream primers were 5'-CATCCGTAAAGACCTCTATGCCAC-3', and downstream primers were: 5'-ATGGAGCCACCGA-TCCACA-3'. The length was within 171 bp. A PCR master mix, containing 4 mM $MgCl_2$, 2.5 U of Taq polymerase and 6 pmol forward and reverse primers, was added to the newly synthesized complementary DNA samples to a total volume of 50 ml. The reactions for PCR amplification were heated to 94°C for 3 min and followed by a re-annealing step at 55°C. The elongation step was performed at 72°C for 60 s. The denaturing-annealing-elongation cycle was repeated 32 times. A 5-min elongation step at 72°C was carried out after the last cycle. The amplified PCR products of the IS and target mRNAs were separated by 2.5% NuSieve/agarose (3:1 w/w)l electrophoresis and visualized by ethidium bromide staining.

Western Blot for PPAR-α Protein Level

Liver samples were homogenized in 20 mM ice-cold HEPES, 1 mM EDTA, and 250 mM sucrose buffer (pH 7.4 with a Polytron (Brinkmann Instruments, Westbury, NY) (12). Protein contents in each sample were quantified by Lowry assay. Equal amounts of proteins were denatured and separated on 7.5% SDS-polyacrylamide gels and then transferred to poly (vinylidene difluoride) membranes (New Life Science Product, Inc., Boston, MA, USA). Nonspecific binding sites on the membranes were blocked with 5% non-fat dry milk in a buffer containing 10 mM Tris-HCl and 100 mM NaCl, pH 7.5, at 4°C overnight. The blots were incubated with PPAR-α and β-actin primary antibodies (1:5000, Sigma, St. Louis, MO, USA) after stripping procedures. PPAR-α protein levels were expressed in relative to β-actin from the same gel. Antigen-antibody complexes were visualized, detected, and quantified with the ECL Western blot detection kit (Amersham Pharmacia Biotech,

Table 2. Polysaccharides and glucans in different strains of *P. tuber-regium*

Components	*P. tuber-regium Strains*		
	MUCL-39359 (High-Percentage, HP)	MUCL-44597 (Medium-Percentage, MP)	MUCL-44822 (Low-Percentage, LP)
Total Polysaccharides (%)	8.18	6.24	3.99
Total Glucan (%)	12.42	4.92	5.23
α-Glucan (%)	6.43	1.09	1.70
β-Glucan (%)	5.99	3.83	3.53

The polysaccharides and glucans contents were expressed in percentage (%) per total dry weight of the each strain of *P. tuber-regium*. β-glucan = total glucan – α-glucan.

Piscataway, NJ, USA), Luminescent Image Analyzer (Fujifilm, Tokyo, Japan), and Zero-Dscan densitometric (Scanalytics, Inc. FairFax, VA, USA) respectively.

Statistical Analysis

All data are represented as the mean ± SD. To evaluate differences among the groups studied, data were analyzed using a one-way analysis of variance (ANOVA) along with Tukey's multiple-range post-hoc test with the SPSS software. $P < 0.05$ was considered statistically significant.

Results

Variance in Polysaccharides and Glucans in Different Strains of P. tuber-regium

The strain MUCL-39359 contain high percentage of total polysaccharides (8.18%) compared to MUCL-44597 (6.24%) and MUCL-44822 (3.99%) strains. Similarly, β-glucans also higher in MUCL-39359 (5.99%) than the other two strains MUCL-44597 (3.83%) and MUCL-44822 (3.53%). Based on the presence of total polysaccharides, strain MUCL-39359 was labeled as "high-percentage polysaccharide (HP)", strain MUCL-44597 labeled as "medium-percentage polysaccharide (MP)" and strain MUCL-44822 considered as "low-percentage polysaccharide (LP)" (Table 2).

Effect of Polysaccharides on Body Weight Changes, Epididymal Fat Weight and Energy Intake

High-fat diet-induced increased body weight in OD group was found to significant ($P < 0.05$) at week 2 and further continued to week 8 compared to CON group. Interestingly, polysaccharides supplementation to OD group showed significantly ($P < 0.01$) decreased body weights from week 4 and continued to week 8. It is noteworthy that the final body weights in polysaccharides supplemented groups approached to CON group (Fig. 1).

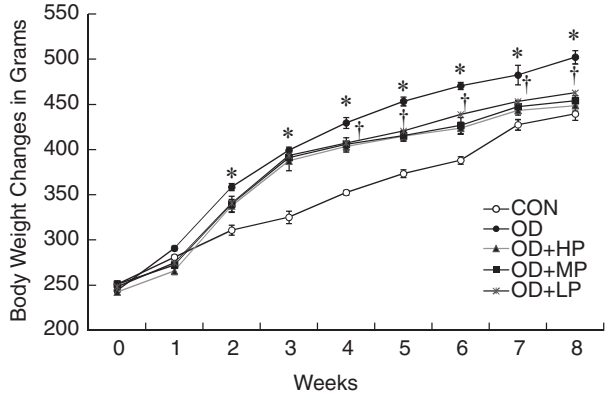

Fig. 1. Changes in body weight (g) over a period of 8 week. CON: normal control with saline, OD: obese-diabetic rats fed high-fat diet plus STZ injections, OD+HP: obese-diabetic rats fed high-fat diet plus STZ along with high-percentage polysaccharides, OD+MP: obese-diabetic rats fed high-fat diet plus STZ along with medium-percentage polysaccharides, and OD+LP: obese-diabetic rats fed high-fat diet plus STZ along with low-percentage polysaccharides. * represents values are significant ($P < 0.01$) compared to CON, and † represents significant ($P < 0.05$) compared to OD groups.

The weight gain in obese-diabetic rats (258 ± 9 g, $P < 0.01$) was greater compared to control (188 ± 5 g) over 8 week period. However, this weight gain was lower in OD+HP (206 ± 7 g), OD+MP (203 ± 7 g) and OD+LP (214 ± 6 g) groups. Similarly, the average epididymal fat weight was significantly ($P < 0.01$) higher in OD rats (16.6 ± 5.0 g) than control (8.2 ± 1.1 g). Statistical analyses reveals significantly ($P < 0.05$) decreased epididymal fat weight by polysaccharides (Table 3). The calculated energy intake was significantly higher in untreated OD rats than control (Table 3). It is note to worth that OD rats treated with high-percentage polysaccharides showed lower energy intake compared to OD untreated rats, while medium- and low-percentage polysaccharides had

Table 3. Weight gain, epididymal fat weight and energy intake in all groups

Groups	Weight Gain (g) (Final – initial weight)	Daily Energy Intake (Kcal/day)	Epididymal Fat (g)	Relative Weight of Epididymal Fat (%)
CON	188 ± 5	111 ± 9	8.2 ± 1.1	1.87 ± 0.15
OD	258 ± 9*	134 ± 7*	16.6 ± 5.0*	3.30 ± 0.72*
OD+HP	206 ± 7†	125 ± 8†	9.1 ± 3.4†	2.03 ± 0.46†
OD+MP	203 ± 7†	132 ± 8	10.6 ± 3.8†	2.33 ± 0.65†
OD+LP	212 ± 6†	130 ± 5	10.8 ± 5.1†	2.33 ± 0.92†

Values are expressed as mean ± SD (n = 10).
CON: normal control with saline, OD: obese-diabetic rats fed high-fat diet plus STZ injections, OD+HP: obese-diabetic rats fed high-fat diet plus STZ along with high-percentage polysaccharides, OD+MP: obese-diabetic rats fed high-fat diet plus STZ along with medium-percentage polysaccharides, and OD+LP: obese-diabetic rats fed high-fat diet plus STZ along with low-percentage polysaccharides. Daily energy intake (Kcal/day) = food intake in g/30 g × 114 or 141 Kcal. Relative weight of epididymal fat (%) = weight of epididymal fat/body weight × 100%.
* represents significant ($P < 0.01$) compared to CON, and † represents significant ($P < 0.05$) compared to OD groups.

Table 4. Blood glucose, serum insulin and adiponectin levels

Groups	Glucose (mg/dl)	Insulin (ng/ml)	Adiponectin (ng/ml)
CON	95 ± 7	1.16 ± 0.13	0.56 ± 0.03
OD	323 ± 2*	0.51 ± 0.12*	0.27 ± 0.01*
OD+HP	216 ± 3*†	1.03 ± 0.15*†	0.41 ± 0.05†
OD+MP	231 ± 2*†	0.82 ± 0.06*†	0.45 ± 0.02†
OD+LP	254 ± 1*†	0.70 ± 0.07*†	0.47 ± 0.01†

Values are expressed as mean ± SD (n = 10).
CON: normal control with saline, OD: obese-diabetic rats fed high-fat diet plus STZ injections, OD+HP: obese-diabetic rats fed high-fat diet plus STZ along with high-percentage polysaccharides, OD+MP: obese-diabetic rats fed high-fat diet plus STZ along with medium-percentage polysaccharides, and OD+LP: obese-diabetic rats fed high-fat diet plus STZ along with low-percentage polysaccharides.
* represents significant ($P < 0.01$) compared to CON, and † represents significant ($P < 0.01$) compared to OD groups.

no effect.

Effect of Polysaccharides on Hyperglycemia and Adiponectin Levels

As a characteristic feature of high-fat diet plus STZ injections, fasting blood glucose levels were enormously increased (240%) at the end of the study in obese-diabetic rats compared to normal control rats. Supplementation of extracellular polysaccharides for 8 week significantly ($P < 0.001$) reduced the hyperglycemia by 33%, 28% and 18% in OD+HP, OD+MP and OD+LP groups respectively. Furthermore, fasting serum insulin levels, which were significantly ($P < 0.001$) decreased were restored by polysaccharides supplementation. Insulin recovery was more prominent in OD+HP group compared to other two strains (Table 4).

The concentrations of adipokines are closely associated with obesity-mediated metabolic syndrome. We found adiponectin levels were significantly ($P < 0.01$) decreased in obese-diabetic rats, but restored by polysaccharides supplementation. Interestingly, adiponectin tended to show an effective response to low-percentage polysaccharides than high- or medium-percentage polysaccharides (Table 4).

Beneficial Effects of Polysaccharides on Hyperlipidemia

Tukey's *post-hoc* test clearly demonstrated that elevated TC, TG and LDL levels ($P < 0.01$) with high-fat diet plus STZ injections were noticeably decreased by polysaccharides supplementation. This antihyperlipidemic property was prominent with high-percentage polysaccharides followed by medium- and low-percentage polysaccharides. Simultaneously, reduced HDL levels in OD untreated rats were tend to recover in all groups treated with extracellular polysaccharides (Table 5).

Effect of Polysaccharides on Hepatic PPAR-α mRNA Expression and Protein Levels

The most important finding of this study indi-

Table 5. Serum total cholesterol (TC), triglycerides (TG), low-density lipoproteins (LDL) and high-density lipoproteins (HDL)

Parameter / Groups	TC (mg/dl)	TG (mg/dl)	LDL (mg/dl)	HDL (mg/dl)
CON	63.8 ± 1.7	56.7 ± 1.7	113.0 ± 4.8	36.6 ± 1.4
OD	157.4 ± 8.6*	199.3 ± 13.6*	168.7 ± 5.2*	25.2 ± 2.2*
OD+HP	112.8 ± 6.5*†	174.0 ± 8.6*†	131.5 ± 1.2*†	35.0 ± 4.5†
OD+MP	129.9 ± 4.2*†	179.4 ± 5.6*†	134.0 ± 1.0*†	31.1 ± 1.3†
OD+LP	133.7 ± 3.4*†	182.6 ± 6.2*†	138.7 ± 1.5*†	30.1 ± 2.5†

Values are expressed as mean ± SD (n = 10).
CON: normal control with saline, OD: obese-diabetic rats fed high-fat diet plus STZ injections, OD+HP: obese-diabetic rats fed high-fat diet plus STZ along with high-percentage polysaccharides, OD+MP: obese-diabetic rats fed high-fat diet plus STZ along with medium-percentage polysaccharides, and OD+LP: obese-diabetic rats fed high-fat diet plus STZ along with low-percentage polysaccharides.
* represents significant ($P < 0.001$) compared to CON, and † represents significant ($P < 0.01$) compared to OD groups.

cated that down-regulated hepatic PPAR-α mRNA expression and decreased protein levels in obese-diabetic untreated rats were significantly ($P < 0.001$) attenuated by polysaccharides. Among three polysaccharide types, PPAR-α mRNA was profoundly up-regulated in OD+HP group (169%), which contains high-percent polysaccharides, and followed by OD+MP and OD+LP groups (Fig. 2A). The augmented PPAR-α protein levels with different polysaccharides was parallel with up-regulated mRNA expression (Fig. 2B).

Influence of Polysaccharides on Plasma and Liver Fatty Acid Composition

As polysaccharides showed promising hypoglycemic and hypolipidemic actions against high-fat diet and STZ injections, we further evaluated the role of polysaccharides on fatty acid composition. The individual fatty acid components in plasma and liver samples were analyzed, and the final values expressed in the form of ratio. Chronic high-fat diet plus STZ-induced elevated n-6/n-3 ratio in plasma and liver has been found to ameliorate ($P < 0.01$) by polysaccharides administration (Table 6). The controlled n-6/n-3 ratio by polysaccharides might be linked with decreased lipid profile as showed in Table 5.

Statistical analyses showed that MUFA/PUFA and MUFA/SPA ratios in plasma and liver samples were significantly dropped ($P < 0.01$) in obese-diabetes group compared to control group. Noticeably, the same parameters were significantly restored by extracellular polysaccharides supplementation that was almost close to the baseline (Table 6).

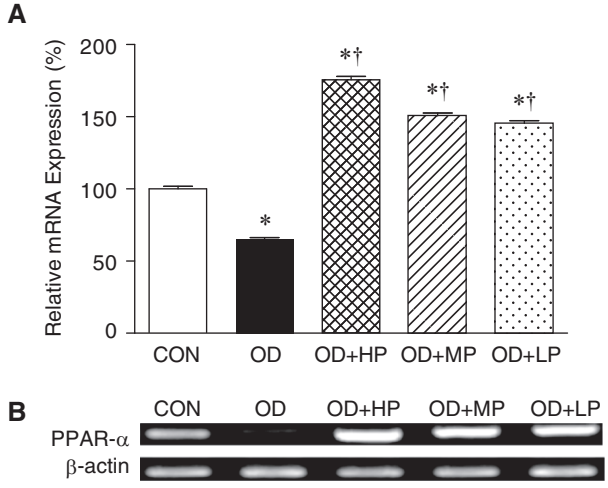

Fig. 2. Liver PPAR-α mRNA expression by RT-PCR (a) and protein levels by western blot (b). CON: normal control with saline, OD: obese-diabetic rats fed high-fat diet plus STZ injections, OD+HP: obese-diabetic rats fed high-fat diet plus STZ along with high-percentage polysaccharides, OD+MP: obese-diabetic rats fed high-fat diet plus STZ along with medium-percentage polysaccharides, and OD+LP: obese-diabetic rats fed high-fat diet plus STZ along with low-percentage polysaccharides. Data were expressed as mean ± SD (n = 3). * represents significant ($P < 0.01$) compared to CON, and † represents significant ($P < 0.01$) compared to OD groups.

Discussion

The high-fat diet plus STZ collectively produce adverse effects on plasma and liver fatty acid composition and decreased hepatic PPAR-α mRNA expression and protein levels. These adverse effects might be associated with increased fundamental features of diabetes progression, including obesity, hyperglycemia and dyslipidemia. However, oral administration of extracellular polysaccharides along with high-fat diet plus STZ injections significantly attenuated the

Table 6. Fatty acid composition (n-6/n-3, MUFA/PUFA. MUFA/SFA ratios) in plasma and liver

Sample Fatty Acid Profile		CON	OD	OD+HP	OD+MP	OD+LP
n-6/n-3	Plasma	13.4 ± 2.1	17.7 ± 2.3*	14.9 ± 2.0†	12.9 ± 1.5†	12.4 ± 1.2†
	Liver	1.25 ± 0.21	1.69 ± 0.12*	1.40 ± 0.10†	1.54 ± 0.13†	1.48 ± 0.14†
MUFA/PUFA	Plasma	0.34 ± 0.02	0.27 ± 0.04*	0.31 ± 0.02†	0.31 ± 0.02†	0.37 ± 0.03†
	Liver	1.80 ± 0.15	1.20 ± 0.21*	1.70 ± 0.04†	1.54 ± 0.13†	1.43 ± 0.17†
MUFA/SFA	Plasma	0.59 ± 0.06	0.33 ± 0.07*	0.48 ± 0.05†	0.48 ± 0.07†	0.67 ± 0.06†
	Liver	0.90 ± 0.02	0.56 ± 0.05*	0.83 ± 0.04†	0.75 ± 0.04†	0.71 ± 0.07†

Values were represented in ratio.

CON: normal control with saline, OD: obese-diabetic rats fed high-fat diet plus STZ injections, OD+HP: obese-diabetic rats fed high-fat diet plus STZ along with high-percentage polysaccharides, OD+MP: obese-diabetic rats fed high-fat diet plus STZ along with medium-percentage polysaccharides, and OD+LP: obese-diabetic rats fed high-fat diet plus STZ along with low-percentage polysaccharides.

* represents significant ($P < 0.01$) compared to CON, and † represents significant ($P < 0.01$) compared to OD groups.

plasma and liver fatty acid composition and promoted the PPAR-α mRNA and protein levels. These beneficial effects may contributed to decrease in the obesity, hyperglycemia and hyperlipidemia, thus polysaccharides may play a vital role in delaying the progression of diabetes.

In our study we developed an experimental obese-diabetes rat model, which mimics the features of metabolic syndrome, including hyperglycemia (diabetes) and hyperlipidemia. The progression of disease was achieved by the combined actions of chronic high-fat diet plus low-dose of STZ injections. Typically, chronic high-fat diet-induced obesity, and low-dose of STZ produced pancreatic β-cell dysfunction are responsible for the occurrence of this obese-diabetes model. Our obese-diabetic rat model was similar with previous experimental diabetic rat models (31, 34, 40).

Hyperglycemia a serious risk factor for CVD was revealed by chronic high-fat diet plus STZ injections. Increased free radicals production and/or damaged β-cells function by high-fat diet plus STZ injections may responsible for the hyperglycemia (36, 37). Thus, administration of antioxidant compounds like polysaccharides may decrease the free radicals-mediated β-cell damage therefore restored the insulin levels (20, 21). On the other hand, increased insulin levels and/or acceleration of glucose metabolism in the liver may contribute to antihyperglycemic property of polysaccharides (27). Polysaccharides are able in improving the insulin secretion by lessening the β-cell damage in experimental diabetic animals (17, 41). Furthermore, polysaccharides can also improve the intestinal microflora production of short chain fatty acid (SCFA) (9), which may inhibit the glucose absorption in intestine. High dietary fiber content in mushrooms may account for the glucose lowering effect (24, 32).

Low levels of adiponectin represent as an independent risk factor for the cluster metabolic syndrome (3). Decreased serum adiponectin levels in OD rats, which were restored by polysaccharides indicates that high-fat diet plus STZ-induced metabolic syndrome could be encountered by polysaccharides. Recently, Inafuku *et al*., (22) reported increased adiponectin levels in db/db mice treated with mushroom extracts. It has been shown that body weight, glucose and lipid metabolisms are regulated by adiponectin (1). Adiponectin concentrations are positively correlated with insulin sensitivity, and plays a key role in insulin action and energy homeostasis (38). Therefore, restored adiponectin by polysaccharides may contribute to decrease the body weights in OD rats.

Deposition of visceral fat is also a primary risk factor in the pathogenesis of various obesity related disorders, including diabetes and hyperlipidemia (14). Our findings further indicated that occurrence of obesity with chronic high-fat diet was effectively lessened by polysaccharides, which may be due to decreased epididymal fat weight. Dietary supplementation of polysaccharides as indigestible fibers may influence the gastrointestinal (GI) functions, including nutrients (fat) absorption, slow gastric emptying and bacterial fermentation in the colon (11). These phenomena may result in reduced calorie uptake. Reduced energy uptake may initiate the mobilization of stored energy from body's fat depots and transport *via* circulation to site of high metabolic demand (35). We assume that polysaccharides that represent for fiber content in mushroom strains may influence the GI functions and facilitate to mobilize the fat depots, thereby decrease the epididymal fat weight.

Hyperglycemia and obesity are closely linked with increased serum cholesterol and triglyceride concentrations. Elevated cholesterol and hyperglycemia was attenuated by mushroom extracts in STZ-diabetic

rat models (20, 24). The cholesterol lowering effect of polysaccharides is as complex process. It has been documented that bioactive components in mushrooms, including SCFA, generated by bacterial fermentation of fibers in colon probably involved in the cholesterol lowering effect (24). In addition, mushroom dietary fibers that contain polysaccharides might bind bile acids to reduce their entry in gut bile acid secretion (6). Consequently, liver responds by increasing the hepatic conversion of cholesterol to bile acids, therefore results in reducing the circulating cholesterol levels (24). Besides, restored HDL levels appeared that β-glucans exists in polysaccharides may participate in removal of LDL from artery and transport mostly to the liver for excretion. Since higher HDL and lower TC and TG concentrations represents least cardiac problems (28), our results implies the cardioprotective properties of polysaccharides.

PPAR-α is a nuclear transcription factor, primarily expressed in liver, which plays an important role in regulation of genes that are involved in lipids utilization and storage and insulin action (2). Up-regulated PPAR-α mRNA by polysaccharides in liver may promote β-oxidation; thereby alleviate the hyperlipidemia-induced liver damage in OD rats. Yamabushitake mushroom extracts has been shown to improve the lipid metabolism, at least in part *via* activation of PPAR-α in mice fed a high-fat diet (18). Up-regulated PPAR-α gene expression by polysaccharides may modulate and facilitate fatty acid oxidation in liver that may results in decreased TG levels. On the other hand, it is also reported that hypoglycemic effect of mushroom polysaccharides possibly through the regulation of PPARs-mediated lipid metabolism (8). Since hyperglycemia and obesity are closely linked with increased serum cholesterol and triglyceride concentrations, therefore this study only examined lipid profiles in serum but not in liver tissues. We suggest that polysaccharide may play as PPAR-α agonists that could decrease plasma TG levels and increase HDL levels by increasing lipid uptake, activation and catabolism through the transcriptional modulation of numerous genes that control these processes (2). Nevertheless, the detailed mechanism behind PPAR-α up-regulation by extracellular polysaccharides considered as complex and remains unclear.

Fatty acids components have been shown to involve in the development of obesity and diabetes (29, 37). In our study, extensive changes of fatty acid composition in plasma and liver by the synergetic impact of high-fat diet plus STZ influenced the obesity and exacerbates to diabetes. However, polysaccharides supplementation along with high-fat diet plus STZ, assisted to keep the stable fatty acid components. It is known that fatty acid, n-6 considered as a risk factor for CVD, while n-3 associated with decreased coronary artery disease (35). Increased intake of n-3 (ω-3) containing food products, such as fish meet shown to prevent the obesity, glucose intolerance (13) and risk of cardiovascular death (12). Therefore, increased n-3 levels, in terms of decreased n-6/n-3 ratio by polysaccharides represents lower risk for CVD.

The increased MUFA/PUFA ratio by polysaccharides further stands for the reduced risk of obese-diabetes associated cardiac threat. Chronic intake of saturated fats certainly affects the membrane fatty acid composition and decrease the metabolic rate by increasing obesity and circulating cholesterol, which progress to CVD (28, 35). High calorie intake has been shown to accelerate fatty acid synthesis, and alter the fatty acid components of MUFA and PUFA in liver (15). In our study, high-fat diet that contains saturated fat (animal oil) decreased the MUFA/SPA ratio in liver and plasma along with increased circulating cholesterol and TG levels. Lower calorie intake by high-percentage polysaccharides (OD+HP) possibly explains the restored MUFA/PUFA ratio. The β-glucans and fiber content in polysaccharides may contribute for this beneficial effect. The exact mechanism behind the polysaccharides beneficial effects is still unclear. However, decreased hyperlipidemia and obesity by polysaccharides, at least in part may contribute to increase the metabolic rate, thereby maintain the stable fatty acid composition.

Our results conclude that extracellular polysaccharides of *P. tuber-regium* are able to attenuate the obese-diabetes-induced adverse effects by maintaining the stable fatty acid composition, and reverting the obesity and hyperlipidemia. These phenomena may support by the up-regulated hepatic PPAR-α mRNA and protein levels, and antihyperglycemic properties of polysaccharides. Since polysaccharides showed the potential beneficial effects on the fundamental feature of diabetes, it is possible to develop a nutraceutical dietary supplement to treat high-fat diet-induced metabolic syndrome.

Acknowledgments

This study was supported by the National Science Council of Taiwan, ROC (NSC99-2324-13-010-001).

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