Original article:

Anti-diabetic effect of Oyster Mushroom mediates through increased AMP-activated protein kinase (AMPK) and cyclic AMP-response element binding (CREB) protein in Type 2 Diabetic model Rats

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Abstract

AMP-activated protein kinase (AMPK) and c-AMP-response element binding protein (CREB) are found to be important proteins in metabolic system. AMPK has become the focus as a novel therapeutic target for the treatment of metabolic syndromes. Oyster mushroom is traditionally used as remedy of diabetes and hypertension. The present study aims to observe the stimulation of AMPK and CREB in streptozotocin-induced diabetic model rats through Oyster mushroom administration. Long Evan's rats were used to create type 2 model diabetic rats through intraperitoneal injection of streptozotocin at 90mg/kg body weight of 48hr old pups. Rats were divided into three groups: diabetic control rats, glibenclamide treated diabetic rats (positive control) and mushroom treated diabetic rats (experimental group). Mushroom was administered orally at a dose of 1.25g/kg body weight in semisolid forms. After five weeks rats were sacrificed, serum and tissues were collected for future analysis. Glucose was measured using glucose-oxidase method, lipid profile by enzymatic-colorimetric method. Proteins from different tissues were extracted using RIPA cell lysis buffer, AMPK and CREB were identified using western blot and immuno-precipitation techniques. A significant decreased of fasting glucose was found after 35 days of experiment when it compared with control diabetic rats (M ± SD, mmol/l, Diabetic control group: 8.0±1.1; Mushroom treated diabetic group: 6.4±1.0; p=0.021). Glibenclamide treated diabetic rats have also shown decreased fasting glucose compared to control diabetic rats. In paired 't' test analysis, it has been found that serum fasting glucose level was significantly decreased on 35th day compared the 0 day in both mushroom treated group (p=0.027) and in glibenclamide treated group (p=0.005). Serum TG level was decreased on 35th day compared to 0day in mushroom treated diabetic model rats only (M±SD, mg/dl, 0 day: 84±13; 35th day: 61±6, p=0.002). No significant changes of cholesterol, HDL and LDL were noticed in the experimental groups following treatment with mushroom. Western blot analyses have shown increased band intensity of AMPK and p-CREB in mushroom treated diabetic model rats. Therefore, it can be concluded that Anti-hyperglycemic property of Oyster mushroom could be explained through increased expression of AMPK and activation of CREB.

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Introduction

Among the major diseases epidemically observed in worldwide, type 2 diabetes (T2D) is considered a significant proportion of them and its prevalence is continues to grow. Untreated and uncontrolled diabetes associates with hypertension, cardiovascular disease, renal failure, nervous system dysfunctioning and certain cancers. It has been projected that there will be about 600 million diabetes people in the world in 2035 and it has been estimated about 5.1

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million deaths caused by diabetes in 2013.[1] Although there is no medications currently available to cure diabetes but several drugs exists for the management of T2D and there is increasing demands for more effective treatments of the disease. AMP-activated protein kinase (AMPK) plays important role in energy homeostasis through increasing cellular glucose uptake in insulin-independent pathway and draws attention as novel drug targets for the treatment of diabetes. Evidences suggest that physical exercise and two major classes of anti-diabetic drugs (biguanides and thiazolidinediones) have recently been reported to activate AMPK[2]. This kinase activates when cellular energy level decreases and functions through downstream stimulation of ATP generation (fatty acid oxidation) as well as inhibit the systems involves in uses of ATP (such as triglyceride and protein synthesis).[3] In associates with adipocytokines like leptin, adiponectin and ghrelin, AMPK also regulates whole-body energy homeostasis through food intake and body weight management. Therefore, AMPK is now regarded as one of the most promising targets for new drugs to treat the growing incidence of metabolic diseases such as obesity and T2D and cardiovascular disease. Crystal structure of core fragments of AMPK has provided essential insights into the mechanisms underlying its allosteric control[4].

Dyslipidemia is associates with both insulin resistance and T2D, and suggested as common risk factor for cardiovascular disease. Diabetic dyslipidemia is a cluster of potentially atherogenic lipid and lipoprotein abnormalities that are metabolically interrelated. AMPK play an important role in the changes of hepatic lipid metabolism and, so, regulates the partitioning of fatty acids through fatty acid oxidation and fatty acid biosynthesis. Upon activation through phosphorylation, AMPK inactivates several metabolic enzymes which functions in ATP-consuming cellular events, such as 3-hydroxy-3-methylglutaryl-coenzyme Areductase (HMG-CoA reductase) and acetyl-CoA carboxylase (ACC), key enzymes in hepatic cholesterol and fatty acid synthesis. In addition, AMPK suppresses expression of lipogenesis-associated genes such as fatty acid synthase, pyruvate kinase and ACC[5-9]. Therefore, AMPK-activating agents would be beneficial for both preventing and treating patients with TD.

The nuclear protein cAMP-responsive element binding protein (CREB), a known transcription factor, required for glucose homeostasis and islet cell survival. Among many other genes CREB regulates beta-cell gene expression, such as the insulin gene and the anti-apoptotic gene *bcl2*. Therefore, CREB is crucial for beta-cell differentiation and survival, the disruption of which leads to metabolic abnormalities and type 2 diabetes[10]. Our findings have also shown that CREB down regulates in streptozotocin-induced T2D model rats.

Mushroom has been traditionally consuming as anti-diabetic and anti-hypertensive plant materials. Although it has also been reported that Oyster Mushroom has anti-diabetic activity[11] but no reports exist where it describes the insight mechanisms. Therefore, the present study attempts to investigate the underline mechanism of anti-diabetic properties of Oyster Mushroom whether it has role on AMPK and CREB.

Methods

Animals: Adult Long Evans rats weighing 130-250g were included in the study. The rats were fed on a standard laboratory pellet diet which contained wheat (40%), wheat bran (20%), rice polishings (5%), fish meal (10%), oil cake (10%), gram (3.9%), pulses (3.9%), milk (3.8%), soyabean oil (1.5%), molasses (0.95%) and salt (0.95%). Embavit GS (vitamin mixture) 250g was added per 100kg of rat food. The experiments were conducted according to the ethical guidelines approved by Bangladesh Association for Laboratory Animal Science.

Type 2 diabetes was induced by a single intra-peritoneal injection of streptozotocin (STZ) in citrate buffer (10ml), at a dose of 90mg/kg of body weight into the rat pups (48 hours old) as described previously[12]. Following 3 months of STZ injection, rats were examined for their blood glucose level by oral glucose tolerance test (OGTT). Diabetic model rats with blood glucose level >7.00 mmol/l, at fasting condition were selected for the study. Oyster mushroom powder was administrated orally to the rats for 35 days at a dose of 1.25g/kg body weight. For all the pharmacological studies, the drug glibenclamide administrated orally at a dose of 5 mg/10 ml/kg body weight for Type 2 model rats. For the control groups, 10ml water was administrated per kg body weight.

Experimental design: A total of 19 type 2 diabetic model rats were used in this 35 days chronic experimental period. They were classified as- **Group-1** (n=6):Type 2 model rats control group, water administered as vehicle [10 ml water/kg body weight], **Group-2** (n=6):Type 2 model rats positive control group, glibenclamide administered as known

anti-hyperglycemic agent[5 mg/10 ml (9.9 ml H_2O + 0.1 ml Twin 20)/kg body weight] and **Group-3** (n=7):Oyster Mushroom treated group (1.25g/kg body weight). Four non-diabetic rats in similar age and weight were also used to compare AMPK and CREB status with diabetic model rats.

Body weight and Blood sample: Body weights of each rat were measured and recorded at seven days interval and accordingly they were fed with their respective treatment.

Blood samples were collected from rats kept under fasting conditions by amputation of the tail tip under diethyl ether anesthesia at 0^{th} and 14^{th} day. The collected blood samples were centrifuged at 2500 rpm for 15 minutes and the serum were separated for biochemical analysis.

Biochemical analysis: Serum glucose was measured by Glucose Oxidase (GOD-POD) method using micro-plate reader (Bio-Tec, ELISA); Serum total cholesterol by enzymatic colorimetric (Cholesterol Oxidase/ Peroxidase) method (Randox Laboratories Ltd., UK), using autoanalyzer, AutoLab; Serum triglyceride (TG) by enzymatic colorimetric method (Randox Laboratories Ltd., UK) using autoanalyzer. Liver glycogen was measured through acid hydrolysis followed by glucose-oxidase method.

Western Blot

Chemicals and Sources: Reagents used in the present study includes RIPA Lysis Buffer, PMSF, Sodium Orthovanadate (Na_3VO_4), Phosphatase Inhibitor, Protein A/G PLUS Agarose bead, protein marker, Goat anti-rabbit IgG-HRP (sc:2054), p-AMPKα 1/2 (Thr 172), AMPKα 1/2 (H-300), p-CREB-1 (Ser-133) and CREB-1 (D-12) were from Santa Cruz Biotechnology (INC), USA. TEMED, Trizma Base and Acrylamide/ Bisacrylamide (29:1) were used from SIGMA Chemical, USA.

Tissue Sample Preparation: Rat tissues from muscle and adipose were homogenized using mechanical force with RIPA (Radio immuno-precipitation assay) lysis buffer under ice-cold condition for 30 minutes and centrifuged for 15 minutes at 12000rpm. Supernatant was collected and assayed for protein content using Bradford protein assay method, 200µg of protein was used for Western Blot and 1500µg for Immuno-precipitation assay.

Protein analysis: Tissue lysates were denatured by boiling for 3 min in Laemmli sample buffer containing 100 mM dithiothreitol. Equal amounts of lysate proteins (200µg of protein per lane) were resolved by SDS-PAGE. For immunoprecipitation, the tissue lysates (1500µg of total protein) were incubated with primary antibody as indicated for 4 h at 4 °C. Immunocomplexes were precipitated from the supernatant with protein A/G-Plus agarose, washed three times with ice-cold cell lysis buffer, and boiled for 3 min in Laemmli sample buffer and resolved by SDS-PAGE. Nitrocellulose membranes were blocked, probed with the specific antibodies, and incubated with horseradish peroxidase-linked secondary antibody followed by enhanced chemiluminescence detection. Visualization and quantification of the bands were obtained using autoradiography and Image J software.

Statistical Analysis: Statistical analysis was performed using Statistical Package for Social Science (SPSS) software for Windows version 16 (SPSS Inc., Chicago, Illinois, USA). Data were expressed as mean±SD. Difference between two groups was assessed by unpaired Student's't' test and paired't' tests as appropriate. A two-tailed p value of <0.05 was considered statistically significant.

Results

Effect of *Oyster Mushroom* on the body weight of type 2 diabetic model rats: Body weight of the rats among the controls, glibenclamide treated rats and mushroom treated rats were similar initially and have not found significant changes among the groups at the end of the experiment.

Table 1: Body weight (g) in different groups of type 2 diabetic model rats during the experimental period

Group	0 Day (M±SD)	7th Day (M±SD)	14h Day (M±SD)	21st Day (M±SD)	35th Day (M±SD)
1 (n=6)	205±27	198±35	202±33	208±39	215±35
2 (n=6)	193±15	185±23	192±17	197±15	190±21
3 (n=6)	201±16	196±33	203±19	204±26	204±26

Group 1, 2 and 3 represent control diabetic model rat, Glibenclamide treated diabetic rat and Mushroom treated diabetic rat respectively. Data presented as mean± standard deviation (M±SD).

Effects of *Oyster Mushroom* on the serum glucose level of type 2 diabetic model rats: To evaluate the effect *Oyster Mushroom* on hyperglycemia, fasting blood glucose was measured on 0, 14 and 35 day of the experimental period. Fasting serum glucose (FSG) levels of type 2 diabetic models rats of different experimental groups were almost similar on 0 day.

In mushroom treated group fasting serum glucose

(M±SD, mmol/L) level was 8.0±2.0, 8.0±1.0 and 6.4±1.0 on 0 day, 14th day and 35th day of the experiment respectively. In glibenclamide treated group fasting glucose concentrations were 8.0±1.0, 7.3±1.1 and 6.0±1.1 on 0 day, 14th day and 35th day respectively. Fasting serum glucose levels on 35th day of group 2 (glibenclamide treated group) and group 3 (mushroom treated group) have found significantly decreased compared to the baseline value.

Table-2: Fasting serum glucose level in different groups of type 2 diabetic model rats 0 day, 14th day and 35th day of the experiment

Group	Fasting serum glucose, mmol/L (M±SD)		
	0 Day	14 Day	35 Day
1	8.1±1.2	8.1±1.1	8.0±1.1
2	8.0±1.0	7.3±1.1	6.0±1.1**
3	8.0±2.0	8.0±1.0	6.4±1.0*

*Group 1, 2 and 3 represent Diabetic control rat, Glibenclamide treated diabetic rat and Mushroom treated diabetic rat respectively. Data presented as mean±standard deviation (M±SD). *p<0.05 compared to group 1; **p<0.01 compared to group 1*

Liver Glycogen levels in different groups of type 2 diabetic model rats: It has been found that liver glycogen (mg/g liver) level was significantly decreased in group 2 (glibenclamide treated group; 12.0±4.1) and group 3 (mushroom treated, 11.0±4.4) compared to the group 1 (control diabetic model rats, 18.2±7.0).

Table-3: Liver Glycogen levels in different groups of type 2 diabetic model rats

Group	Glycogen (mg/g) (M±SD)
1	18.2±7.0
2	12.0±4.1
3	11.0±4.4*

*Data presented as mean±standard deviation (M±SD). *p<0.05 in unpaired student's 't' test compared to group 1.*

Effects of *Oyster Mushroom* on the Lipid profile of type 2 diabetic model rats

Chronic effect of ***Oyster Mushroom*** on lipid profile of type 2 diabetic model rats have been depicted in table 4. In paired 't' test, serum TG level in mushroom treated rats have shown significantly deceased on 35th day compared to 0 day, no such differences have observed for cholesterol, HDL or LDL.

Effect of Oyster Mushroom on the phospho-AMPK (Thr 172) protein in muscle and adipose tissue of type 2 diabetic model rats

In figure 1, p-AMPK from muscle and adipose tissues of non-diabetic, diabetic and mushroom treated diabetic rats have been detected between the marker protein of 85KDa and 50KDa. P-AMPK seems to be decreased in STZ-induced diabetic model rats (lane 3, 4) compared to the non-diabetic rats (lane 1, 2). Both the muscular and adipose tissue protein of p-AMPK were almost similar in mushroom treated diabetic rat (lane 5, 6) as shown in diabetic rats.

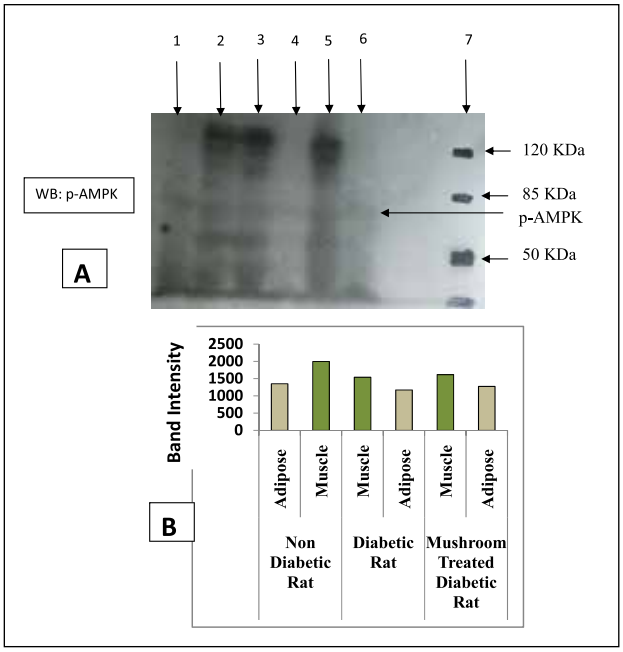

Figure 1: Effect of Oyster Mushroom on the phospho-AMPK, A. 200 µg of proteins from different tissues of rats were analyzed using western blot. **Lane 1, 2:** Muscle and adipose tissues of non-diabetic rat respectively; **lane 3, 4:** Muscle and adipose tissues of diabetic rat; **lane 5, 6:** Muscle and adipose tissues of mushroom treated diabetic rat respectively**; lane 7:** Protein marker. **B.** Graphical presentation of band intensity of p-AMPK

Effect of Oyster Mushroom on the AMPK protein contents in muscle and adipose tissue of type 2 diabetic model rats

In figure 2, total AMPK from muscle and adipose tissue have been presented from diabetic, non-diabetic and mushroom treated diabetic rats as shown in the figure. AMPK seems to be decreased in muscle and pancreas tissues of STZ-induced diabetic model rats (lane 5, 7) compared to the non-diabetic rats (lane 2, 4). The muscular and pancreatic tissue protein of AMPK were increased in mushroom treated diabetic rat (lane 8, 10) compared to the non-

Table-4: Serum lipid profile levels between 0 day and 35th day in different groups of type 2 diabetic model rats (paired 't' test)

Group	TG (mg/dl) (M±SD)		Cholesterol (mg/dl) (M±SD)		HDL (mg/dl) (M±SD)		LDL (mg/dl) (M±SD)	
	0 Day	35 Day	0 Day	35 Day	0 Day	35 Day	0 Day	35 Day
1	64±17	66±11	66±7	66±14	40±4	35±5	17±7	29±12
2	62±14	57±15	66±6	59±11	38±6	36±7	17±4	17±6
3	84±13	61±6**	67±9	68±8	38±4	35±6	16±6	20±6

*Group 1, 2 and 3 represent Diabetic control rat, Glibenclamide treated diabetic rat and Mushroom treated diabetic rat respectively. TG, triglyceride; Chol, Cholesterol; HDL, High density lipoprotein; LDL, Low density lipoprotein. Data presented as mean± standard deviation (M±SD). **p<0.01 compared to baseline value.*

treated diabetic rat (lane 5, 7). No changes of AMPK protein expression has observed in adipose tissues of diabetic, non-diabetic or treated rats.

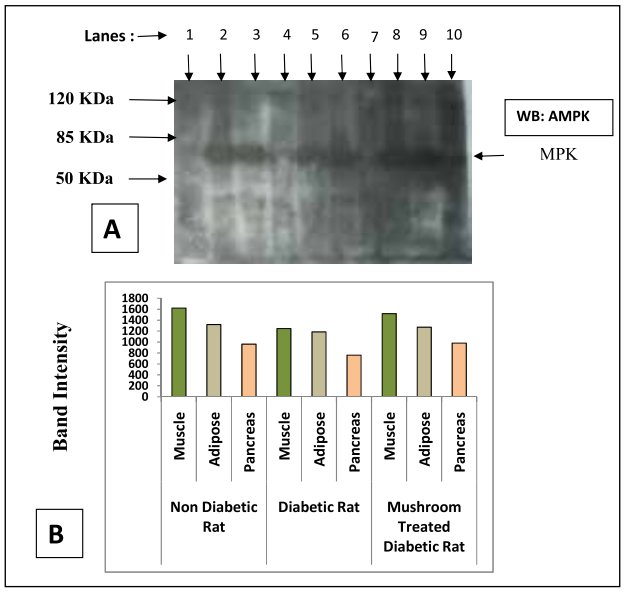

Figure 2: Effect of Oyster Mushroom on the AMPK protein expression, A. 200 µg of proteins from different tissues of rats were analyzed using western blot. **Lane 1:** Protein marker; **Lane 2, 3, 4:** Muscle, adipose and pancreas tissues of non-diabetic rat respectively; **lane 5, 6, 7:** Muscle, adipose and pancreas tissues of diabetic rat; **lane 8, 9, 10:** Muscle, adipose and pancreas tissues of mushroom treated diabetic rat respectively. **B.** Graphical presentation of band intensity of AMPK

Effect of Oyster Mushroom on the phospho-Cyclic AMP Response Element Binding Protein (p-CREB) contents in muscle tissue of type 2 diabetic model rats

In figure 3, p-CREB has been presented around the position of 50 KDa. P-CREB seems to be decreased in STZ-induced diabetic model rats (lane 4, 5) compared to the non-diabetic rats (lane 2, 3). The muscular protein of p-CREB were increased in mushroom treated diabetic rats (lane 6,7,8 and 9) compared to the non-treated diabetic rat.

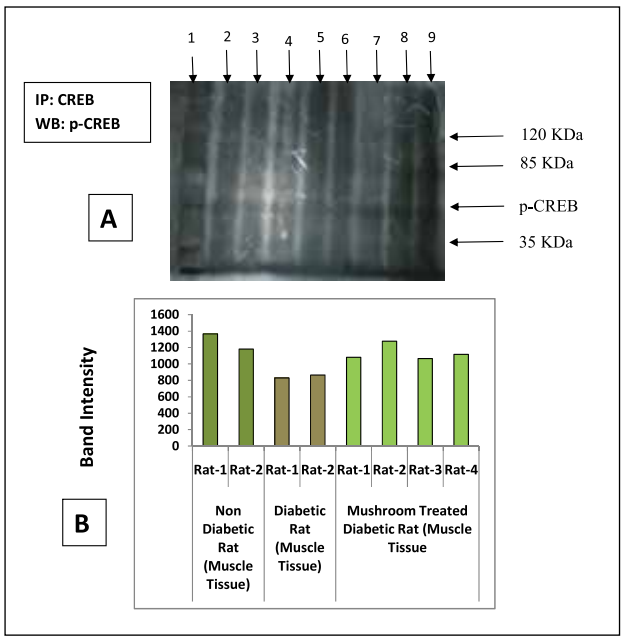

Figure 3: Effect of Oyster Mushroom on p-CREB, A. 1500 µg proteins from muscle tissues of rats were immunoprecipitated against anti-CREB antibody with Protein A/G Plus agarose beads followed by western blot against anti-p-CREB antibody. **Lane 1:** Protein marker; lane **2, 3:** Muscle tissues of non-diabetic rat respectively; **lane 4, 5:** Muscle tissues of diabetic rat; **lane 6, 7, 8, 9:** Muscle tissues of four mushroom treated diabetic rat respectively**. B.** Graphical presentation of band intensity of p-CREB.

Discussion

Regulation of energy metabolism involves Adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) and placed the center stage in studies of diabetes and related metabolic diseases. AMPK

expressed in all the metabolically relevant organs and is activated in response to a variety of stimuli like agents that exert impacts on cellular metabolism, several hormones, cellular stress and exercise[13]. Hyper-triglyceridaemia and high cholesterol which are the potential risk factors for cardiovascular problems are generally associates with T2D. AMPK, after activation, could reduce the risk of cardiovascular diseases through management of elevated level of TG, cholesterol and free fatty acids[14]. Pharmacological activation of AMPK have improves hyperglycemia, dyslipidemia and hypertension in insulin resistant model rodents. Therefore, searching of pharmacological agents that activates AMPK is drawing key attention for novel therapeutic target as unique challenge in the treatment of type 2 diabetes.[13]

Pleurotus is a genus of gilled mushrooms which includes one of the most widely eaten mushrooms, *P. ostreatus*. Species of *Pleurotus* may be called **oyster**, **abalone**, or **tree mushrooms**, and are some of the most commonly cultivated edible mushrooms in the world[15]. It contains 15-35% protein content (equivalent to an egg or 1/3 of meats). It lowers lipid concentrations[16]. This mushroom also reduces blood glucose levels[10] and has antioxidant effect to prevent aging of major body organs[17,18].

Based on the existing evidences of anti-diabetic properties of Oyster mushroom the present study aims to observe the effects of this herb on activation of proteins involves in insulin signaling pathway specially on the activation of AMPK and related kinase in STZ-induced diabetic model rats.

Streptozotocin inhibits oxidative mechanisms in cellular metabolic pathways and is well known for cytotoxicity in pancreatic islet β-cell.[21,22] Experimental and clinical studies have shown that the oxidative stress plays a major role in the development and progression of both types of diabetes.[21,22] In the present study STZ-induced diabetic rats were chosen as the animal model because it resembles many of the features of human T2D[23].

The results of the present study showed that the oral administration of Oyster mushroom for 35 days significantly ($p=0.002$) decreased the levels of fasting blood glucose in STZ-induced diabetic rats as found in human study[12]. The effects of oyster mushroom on diabetic complications associated with dyslipidemia were assessed by measuring the atherogenic lipids (i.e., cholesterol and triglycerides) after chronic feeding of Oyster mushroom to diabetic rats. The obtained results demonstrated that serum TG level was significantly decreased in mushroom treated group but no significant changes have found on serum cholesterol, HDL or LDL.

Liver glycogen level may be considered as the best marker for assessing hypoglycemic activity of any drug. It is usual that peripheral free glucose is being store in the liver in the form of glycogen by increasing glycogenesis. In diabetes this situation aggravates since there is more glucose in the blood to be stored in liver. Glycogen is the molecule that functions as the long-term energy storage in animal cells. Hypoglycemic agents increase the glucose uptakes in both muscle and adipose tissue to generate energy and left less for liver in glycogenesis. In our study we have found that both the glibenclamide and mushroom treated group have shown decreased levels of liver glycogen compared to the non-treated diabetic rats which is the normal characteristics of hypoglycemic agents.

The major objective of this study was to investigate the effect of oyster mushroom on the activation of AMPK signaling pathway. Metformin, a hypoglycemic agent acts by increasing the phosphorylation and activation of AMP-activated protein kinase, a key enzyme involved in the regulation of gene expression, fuel metabolism, and energy balance[24]. This study have documented that p-AMPK which is reduced in STZ-induced diabetic rats have slightly improved after 5 weeks treatment with Oyster mushroom. The study has also shown that muscle tissue produces more p-AMPK compared to adipose tissues of Long Evan's rat. This study fails to detect p-AMPK in pancreas, no study exists which documented the p-AMPK in rat pancreatic tissues. When we have looked about the total AMPK status using western blot analysis of tissue proteins, it has been shown that expression AMPK was reduced in STZ-induced diabetic rats whereas the mushroom treated rats seems to have improved the AMPK levels as in non-diabetic rats. AMPK activation results in increased glucose uptake in skeletal muscle[25] but decreased hepatic glucose production. AMPK regulates glucose both via the direct phosphorylation of metabolic enzymes and effects on gene expression[26]. Although Oyster mushroom have not shown increased phosphorylation of AMPK but increased expression of AMPK and this may helps to regulates glucose metabolism through increased expression of other genes.

CREB (cAMP-response element binding protein), a nuclear protein acts as a transcription factor after phosphorylation (p-CREB) and may helps to synthesis insulin. In pancreatic beta-cell line, it has

been shown that high glucose treatment for 24hr decreased the activation of CREB and also decreased the secretion of insulin[27]. In our animal experiment we have found that p-CREB was reduced in STZ-induced diabetic rats whereas in mushroom treated diabetic rats improves the activation of CREB. Therefore, we could speculate that Oyster mushroom may helps to regulate glucose metabolism by increased insulin signaling pathway through CREB activation.

Conclusions

The above discussion may demonstrate that Anti-hyperglycemic property of Oyster mushroom could be explained through higher expression of AMPK and activation of CREB.

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