Effects of Dietary Supplementation with *Agaricus sylvaticus* Schaeffer on Glycemia and Cholesterol after Streptozotocin-Induced Diabetes in Rats

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This study evaluated the effect of the *Agaricus sylvaticus* (sun mushroom) on biochemical tests of the plasma and on the morphology of the pancreas in an experimental model of type I diabetes mellitus (DMI) induced by streptozotocin. One gram of dry *A. sylvaticus* was homogenized and mixed with the chow. Male Wistar rats were allocated as follows: normoglycemic control that received commercial chow; normoglycemic control group that received chow with *A. sylvaticus*; diabetic group that received commercial chow; and diabetic group that received chow with *A. sylvaticus*. Weight, food, and water consumption were measured every two days. Blood glucose levels were measured twice a week. After 30 days, the animals were euthanized and blood was collected for the analysis of cholesterol, HDL, triglycerides, blood sugar, glutamic-pyruvic transaminase (GPT), alkaline phosphatase, iron, transferrin, and urea. The pancreas was processed for microscopic analysis. *A. sylvaticus* modulated the levels of cholesterol, HDL, triglycerides, blood sugar, GPT, alkaline phosphatase, iron, transferrin, and urea to levels similar to those found in the controls and led to compensatory hyperplasia of the islets of Langerhans. *A. sylvaticus* is potentially beneficial in the control of type 1 diabetes, and it may also prevent pancreas damage.

1. Introduction

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from deficient insulin secretion and/or action [1]. This disease is associated with a reduction in quality of life and an increase in risk factors for comorbidities and mortality. Long-term hyperglycemia is an important factor to the development and progression of microvascular and macrovascular complications [2, 3].

A number of natural products have been employed for the prevention and treatment of diseases. According to the World Health Organization (WHO), nearly 80% of the population worldwide relies on medicinal herbs as their primary form of health care [4]. Among such products, mushrooms, especially basidiomycetous fungi, have attracted attention due to their low toxicity and high degree of specificity in the activation of the human immune system [4].

The basidiomycete *Agaricus blazei* Murrill is currently cultivated in different regions of Brazil [5]. Similar mushrooms as *Agaricus sylvaticus* (Schaeffer) and *Agaricus brasiliensis* [6] are equally distributed on the country. These three species of mushrooms are popularly denominated “sun mushrooms” [5, 7, 8].

The qualitative characteristics of mushrooms are influenced by the species, strain, postharvest processing, age, environment, and locality [7]. Oliveira et al. (1999) [9] analyzed the nutritional value of *A. blazei* and concluded that this mushroom is rich in proteins (30%), fibers (14.6%), and minerals (P = 2.34%; Ca = 0.07%; S = 0.29%; Zn = 86.9 mcg; Fe = 79.13 mcg) and has a low content of lipids (1.48%).
Subsequent studies report different figures, but all agree that the sun mushroom has a rich chemical composition with a wide variety and quantity of minerals and protein [10–12].

Agaricus blazei is rich in immunomodulating polysaccharides and β-glucans and has been shown to have antitumor, anti-infection, antiallergic, and antiasthmatic properties in mice models in addition to anti-inflammatory effects in individuals with inflammatory bowel disease [13, 14]. β-Glucan extracts from A. blazei have been reported to reduce blood glucose, triglycerides, and cholesterol levels and exhibit insulin-like action. To clarify the antidiabetic efficacy and hypoglycemic mechanisms, Niwa et al. (2011) [14] submitted rats with streptozotocin-induced diabetes to daily oral feeding with powdered sweet potato (Ipomoea batatas) and A. blazei and found a decrease in the fasting plasma glucose and glycated hemoglobin as well as the restoration of body weight loss due to diabetes. The authors concluded that the hypoglycemic effects of these products result from the suppression of oxidative stress and proinflammatory cytokine production, followed by an improvement in pancreatic β-cell mass.

The aim of the present study was to evaluate the effect of Agaricus sylvaticus on the blood sugar, cholesterol, HDL, triglycerides, glutamic-pyruvic transaminase (GPT), alkaline phosphatase, iron, transferrin, and urea level in the plasma of rats with type 1 diabetes mellitus (DMI) induced by streptozotocin. Moreover, a morphological analysis of the pancreas of the animals was made.

2. Materials and Methods

2.1. Preparation of Agaricus sylvaticus and Analysis. A. sylvaticus was cultivated in the state of Rio Grande do Sul (Brazil). After harvesting, the fruiting bodies were washed in water, dried, and finely milled in a hammer mill equipped with a 1 mm mesh stainless steel sieve (Vitali Cogumelos, Rio Grande do Sul, Brazil). The sifted powder was stored dry until further use. Dry weight was normally between 7 and 10% of fresh weight. One gram of dry mushroom was homogenized in 40 mL of water, maintained at 4°C for one hour, and filtered with Whatman number 1 filter paper.

The extract was read in a spectrophotometer UV/light 270–580 nm (Beckman DU 530, Beckmann Instruments Inc.) and 1 mL was submitted to Sephadex G-50 chromatography gel filtration (2.0 × 10 cm) at 25°C. Elution was performed with phosphate buffer (10 mmol/L, pH 7). Flow was 2 mL for 1 minute and samples were collected in 20 tubes. Pooled fractions were submitted to the analysis of proteins [15] and phenols [16]. Lever’s method was used for beta-glucan [17], which involved 0.1 mL of extract diluted in 50 mM sodium acetate, pH 5, mixed with 0.1 mL of beta-glucanase enzyme (Sigma), and kept for 30 min at 37°C. At the end of the reaction, 1.5 mL of p-hydroxybenzoic acid hydrazide (Sigma) was added. The samples were heated to 100°C for 10 min and cooled to room temperature and absorbance was read at 410 nm. Optical density was converted into mg of reducing sugar using a standard curve prepared with glucose and laminarin (Sigma). One glucanase unit is defined as the amount of enzyme that produces 1 mg/mL from laminarin glucose. Insoluble beta-glucan was calculated in a sample without enzyme.

Thin-layer chromatography was performed on Merck silica gel 60 F254 plates (10 cm × 10 cm). Aliquots of standard p-coumaric acid, kaempferol, chlorogenic acid, salicylic acid, rutin and the extracts were applied as spots at the origin on a plate and developed with butanol-acetic acid-water (BAW 4:1:5) in a presaturated chromatographic chamber. Developed plates were dried with a stream of hot air (hair dryer) and viewed with ultraviolet (UV) light and ferric chloride (1% in alcohol). The Biorad software program was used to determine the area of each spot on the plate. Each Rf value was compared to a curve prepared using the standard p-coumaric acid (Rf = 0.88), kaempferol (Rf = 0.94), chlorogenic acid (Rf = 0.52), salicylic acid (Rf = 0.77), and rutin (Rf = 0.48) using the standard (10 μL of each solution) p-coumaric acid. The extract was also quantified in a UV/light 275–600 nm spectrophotometer (Beckman DU 530–Beckmann Instruments Inc.), with the readings compared to the peak absorbance of the standards.

Based on the results of the biochemical analysis, 1 g of mushroom powder was extracted with 40 mL of cool water and mixed with 9 g of previously crushed, sieved commercial feed, forming round pieces, and placed in a Pyrex container. All samples were dried in an oven at 60°C. The chow was then weighed and placed in the animal cages. One gram of feed with A. sylvaticus was diluted in 2 mL of water and the quantity of beta-glucan was then analyzed.

One gram of the commercial chow (Bio Base, São Paulo, SP, Brazil) contained protein (22 mg), calcium (1 mg), phosphorus (0.8% mg), fiber (0.8 mg from rice bran, wheat, and corn), minerals (1 mg), and 12% moisture.

2.2. Animals. Male Wistar rats aged four weeks and weighing 250 to 280 g were obtained from the University Nove de Junho (UNINOVE) animal lodging facility. The animals were kept in polypropylene cages (two to three animals per cage) covered with metallic grids in a room maintained at 23°C, 55±10% relative humidity, and a 12 h light/dark cycle. The animals had free access to food and water for two weeks before beginning the study. The UNINOVE Ethics Committee for Animal Research approved the protocols used in this study (process numbers: 34/2010 and 20/2012).

2.3. Diabetes Type 1 Induction and Experimental Groups. The animals were divided into four groups of five rats. In two groups, the animals were fasted for 12 hours and chemical diabetes was induced through an intraperitoneal injection of streptozotocin (50 mg/kg) (STZ, Sigma). The STZ solution was prepared immediately prior to injection by dissolving the drug in a fresh, cold citrate buffer, pH 4.5. After 72 hours, blood glucose levels were measured using a portable glucose meter (One Touch II; Johnson & Johnson, Milpitas, CA). For such, the distal part of the tail was gently snipped; the first blood drop was discarded and the second was absorbed by a test strip inserted in the glucose meter. Rats were considered diabetic when the blood glucose level was at least 250 mg/dL.
The groups were evaluated for 30 days. Food and water consumption were measured every two days. Blood glucose levels and weight were measured twice a week, always at 11:00 a.m.

2.4. Euthanasia and Sample Collection. At the end of the experimental period, the animals were anesthetized with a lethal dose of a cocktail containing ketamine (1 mg), xylazine (5 mg), and acepromazine (0.2 mg). Thoracotomy was performed. Blood was collected from the left ventricle and centrifuged. The plasma was removed and stored at $-20^\circ$C for no longer than three days before the assay. Total cholesterol, triglycerides, HDL cholesterol, urea, creatinine, iron, and transferrin were measured using test kits (Labtest Diagnostica).

2.5. Histological Analysis. After euthanasia, the pancreas was removed, rinsed with water, fixed in a 10% buffered formalin solution, and analyzed in paraffin wax. Sections measuring $5\mu m$ in thickness were prepared and stained with hematoxylin and eosin [18].

The three best sections from each animal were submitted to microscopic examination. Five consecutive areas of each sample were photographed (Leica Microsystems, Wetzlar, Germany) (160x magnification) and analyzed using the Image J program with the “measure” plug-in (version 1.45q, free software, NIH, Bethesda, Maryland, USA) for the measurement of the islets of Langerhans. The number of islets and nuclei in each islet were also counted.

2.6. Statistical Analysis. Statistical analysis was performed using the Assistat-2012 software and involved ANOVA, the Student's $t$-test, and Tukey's test. Statistical significance was determined by $P$ values <0.05 and <0.01.

3. Results

3.1. Analysis of A. sylvaticus. The fruiting bodies had a light yellow color, indicating that drying was carried out at 40 to 60$^\circ$C to avoid caramelizing the product. The finely milled powder demonstrated solubility in water and was therefore used in the present study.

For the biochemical analysis, the extract from A. sylvaticus was made with cool water to extract the polysaccharide bound to protein without the action of phenols or peroxidase activity, thereby obtaining an extract with a light yellow color. When hot water was used, more phenols were extracted and the protein was decreased, giving a brown coloration to the solution corresponding to the oxidation of molecules [8].

The extract was first submitted to UV/light spectrophotometry at wavelengths of 275 to 600 nm, demonstrating three peaks: (1) a peak at A280 nm correlated with proteins; (2) a peak at A340 nm correlated with chlorogenic acid and p-coumaric acid; (3) peaks at A500 nm that were not identified. Phenols were confirmed by thin-layer chromatography using BAW (4:1:5) and the results demonstrated four compounds: $R_f = 0.29$ (unknown identity), $R_f = 0.52$ (correlated with the chlorogenic acid standard), $R_f = 0.68$ (unknown identity), and $R_f = 0.88$ (correlated with p-coumaric acid) (Figure 1).

The biochemical analysis of the mushroom powder revealed 1.15 mg of proteins, 0.96 mg of phenols, 224.03 mg of beta-glucan, 31 mg of fiber, p-coumaric acid, chlorogenic acid, and two unknown compounds.

One mL of crude extract was submitted to chromatography gel filtration and separated into four pooled fractions: (1) external volume (fraction tubes 3 and 4), (2) internal volume (tubes 6, 7, and 8), (3) tubes 10, 11, and 12, and (4) tubes 13, 14, 15, and 16. All fractions were submitted to analyses for the quantification of protein, phenols, and beta-glucan (Figure 2).
3.2. Animal Weight, Food, and Water Consumption. In the control groups (C and CA), weight increased throughout the experimental period, with no significant differences between the two groups. Significant differences were found in the comparison of C with the D and DA groups ($P < 0.05$). Weight in the D group remained largely stable throughout the period, whereas a slight decrease was found in the DA group. Moreover, a significant difference was found between the D and DA groups (Figure 3). Interestingly, while the animals in the C and CA groups had similar increases in weight, the CA group consumed a significantly greater amount of food than the C group. As expected, food and water consumption were greater in the D group. However, when the diabetic rats began to consume *A. sylvaticus* (DA group), food and water intake decreased (Figures 4 and 5).

3.3. Biochemical Analysis of Plasma. Table 1 displays the results of the biochemical tests at the time of euthanasia.

3.3.1. Kidney Function Markers: Creatinine and Urea. A significant increase was found in serum urea between the D group and the other groups. Moreover, serum urea was significantly lower in the DA group when compared to the D group ($P < 0.01$). In contrast, no significant differences among groups were found regarding serum creatinine (Table 1).

3.3.2. Markers of Tissue Oxygenation: Transferrin and Iron. Significant reductions were found in serum transferrin and iron between the D group and the other groups, whereas no significant differences were found among the C, CA, and DA groups (Table 1).

3.3.3. Markers of Liver Function: Alkaline Phosphatase and Glutamic-Pyruvic Transaminase (GPT). The alkaline phosphatase level in the D group was strongly elevated in comparison to the other groups, whereas no significant differences were found among the C, CA, and DA groups. The same pattern was found with regard to GPT (Table 1).

3.3.4. Total Cholesterol, HDL Cholesterol, and Triglycerides. Significant increases in serum cholesterol and triglycerides and a significant reduction in HDL were found in the D group in comparison to the other groups. The DA group exhibited a reduction in triglycerides and total cholesterol as well as an increase in HDL. A slight decrease in cholesterol and increase in HDL level were found in the DA group in comparison to the C and CA groups (Table 1).

3.3.5. Blood Glucose Levels. One day before the beginning of the experiment, serum glucose concentration was within the normal range (82 to 122 mg/dL). Three days after diabetes induction, increases in glucose concentration were found in
Figure 4: Food consumption of rats submitted to treatments (C: normoglycemic control group having received unaltered commercial chow; CA: normoglycemic control group having received chow with A. sylvaticus; D: diabetic group having received unaltered commercial chow; DA: diabetic group having received commercial chow with A. sylvaticus). Measurements were taken every two days for 4 weeks. Values are mean of 5 animals. “∗” on lines indicates statistically significant differences between the group and the control ($P < 0.05$; ANOVA + Tukey’s test).

Figure 5: Water consumption of rats submitted to treatments (C: normoglycemic control group having received unaltered commercial chow; CA: normoglycemic control group having received chow with A. sylvaticus; D: diabetic group having received unaltered commercial chow; DA: diabetic group having received commercial chow with A. sylvaticus). Measurements were taken every two days for 4 weeks. Values (mL) are mean of 5 animals. “∗∗” on lines indicates statistically significant differences among all groups ($P < 0.05$; ANOVA + Tukey’s test).

Figure 6: Glycemia in rats submitted to treatments (C: normoglycemic control group having received unaltered commercial chow; CA: normoglycemic control group having received chow with A. sylvaticus; D: diabetic group having received unaltered commercial chow; DA: diabetic group having received commercial chow with A. sylvaticus). Measurements were taken twice a week for 4 weeks. Values are mean of 5 animals. The first measurement is prior to the initiation of the experiment (time zero). “∗∗∗” on lines indicates statistically significant differences among all groups ($P < 0.05$; ANOVA + Tukey’s test).

Figure 7:Histopathological Analysis. In both control groups (C and CA), islets of Langerhans were present with normal size and shape. The measurements demonstrated that both control groups had a similar mean area of islets and similar mean number of nuclei (410.5 $\mu m^2$ and 57 nuclei per islet). The samples from the D group exhibited severe degenerative changes in the pancreatic islets, with irregular outlines and decreased nuclei in $\beta$-cells (mean islet area: 0.62 $\mu m^2$; mean number of nuclei: 20) showing a significant decrease in size in comparison to control group and Agaricus group (Kruskal-Wallis test, $P < 0.05$). In contrast, the DA group exhibited nearly normal pancreatic islets slightly oval in shape (mean area: 254.7 $\mu m^2$; mean number of nuclei: 87) (Figure 7 and Table 2).

4. Discussion

The present study demonstrates that rats with type 1 diabetes induced by streptozotocin showed elevated levels of cholesterol, HDL, triglycerides, blood glucose, GPT, alkaline phosphatase, and urea and reduced levels of iron and transferrin 30 days following exposure. Dietary supplementation of standard chow with Agaricus sylvaticus Schaeffer extracts attenuated these metabolic changes in DMI animals. Otherwise, normoglycemic animals exhibited no metabolic changes with the use of A. sylvaticus. The findings suggest a protective effect of A. sylvaticus on the pancreas and in other metabolic biochemical parameters toward homeostasis without change in the nondiabetic individuals that were already in a state of equilibrium (homeostasis).

In the present study, powdered A. sylvaticus from the state of Rio Grande do Sul, Brazil, demonstrated complex compounds and proved water soluble. One gram of fungus in 40 mL of water liberated beta-glucan (224.03 mg), proteins...
(1.15 mg), phenols (0.96 mg), p-coumaric acid, chlorogenic acid, and nonsoluble substances, such as fiber (31 mg) [7].

Many fibers have a polysaccharide β-glucan, which is found in a large amount in the cell wall of fungi. Dietary fibers have been strongly implicated in the prevention and treatment of various characteristics of metabolic syndrome. Epidemiologic studies have consistently reported an inverse relationship between dietary fiber and both type 1 and type 2 diabetes mellitus and cardiovascular mortality [19–25]. Babio et al. (2010) examined the effect of different types and sources of dietary fiber on body weight, glucose metabolism, and lipid profile and concluded that the intake of viscous dietary fiber decreases low-density lipoprotein cholesterol and postprandial glucose levels and induces short-term satiety [2].

A number of mechanisms have been suggested to explain the reductions in blood glucose and lipids in the blood as well as body weight through the ingestion of soluble beta-glucan and insoluble fibers. One such mechanism involves the ability of soluble fibers to form viscous solutions. The effects occur in the gastrointestinal tract, with an improvement in lassation and an increase in stool bulk due to the inhibition of the formation of fat in the organism, with metabolic consequences, including improved health [26–28]. In our experiments, while the animals in the C and CA groups had similar increases in weight, the CA group consumed a significantly greater amount of food than the C group, so our results are in accordance with the literature.

The type 1 diabetes model chosen for this study was the use of the chemotherapeutic drug streptozotocin. STZ is selectively toxic to β-cells in pancreatic islets and eventually induces an inflammatory process in pancreas producing diabetes in adult rats. STZ-induced hyperglycemia has been described as a good experimental model for the study of DMI [29].

A possible explanation for the findings with regard to metabolic control by Agaricus blazei may reside in the quantity and structure of β-glucan in this mushroom. The cell wall β-glucans of yeast and fungi consist of 1,3 β-linked glucopyranosyl residues, with small numbers of 1,6 β-linked branches. These characteristics may influence the immune-modulating effects [12, 19] and can generate an anti-inflammatory response influencing IL-6, prostaglandin D2, and leukotriene C4 [30].

Moreover, signal mediation is a possible mechanism for the reduction of blood glucose by beta-glucans. Insulin needs to bind to a receptor, which is composed of two extracellular alpha and two transmembrane beta subunits. After the binding of insulin to the extracellular subunit, the intracellular subunit tyrosine kinase is activated [31]. Consequently, four members of the insulin receptor substrate (IRS) family are activated. A phosphorylated reaction from IRS creates sites for binding to another proteins equivalent to phosphatidylinositol 3-kinase (PI3K) with serine/threonine kinase B (Akt). The PI3K/Akt pathway regulates a large
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number of cell functions, such as apoptosis, cell growth, metabolic effects stimulated by insulin, and the inflammatory process. Decreased PI3K/Akt activity has been shown to play a key role in the pathogenesis of diabetes. Beta-glucans have been demonstrated to increase PI3K/Akt through different receptors [32, 33].

The morphological evaluation of the pancreatic islets revealed compensatory hyperplasia induced by *A. sylvaticus*. This finding suggests a stimulatory effect on these cells. Niwa et al. used the same experimental model in a DM1 study and found similar results with *Agaricus blazei* [36]. Moreover, the mushroom has a percentage of the soluble fiber beta-glucan, which may help maintain blood sugar, normal lipid content, and normal blood pressure are among the goals of medical treatment in such patients.

Water intake was similar in the two control groups (C and CA), but food intake was greater in the CA group. Nonetheless, body weight was maintained in this group. The fact that the CA group ingested more food indicates the palatability was unchanged with the addition of *A. sylvaticus*. Palatability is a main factor that influences food intake by animals [36]. Moreover, the mushroom has a percentage of the soluble fiber beta-glucan, which may help maintain body weight. Depending on the physicochemical properties, fibers have a range of physiological consequences, including viscosity in the upper gastrointestinal tract [37], fermentation in the colon [38], and prebiotic effects [39, 40].

High blood sugar levels can place stress on the kidneys, which can cause serious damage to blood vessels. High concentrations of creatinine and urea constitute markers of kidney dysfunction [41, 42]. In the present study, the animals in the D group exhibited increased urea in comparison to the control groups (C, CA), indicating that diabetes can lead to kidney dysfunction. The significantly lower level of serum urea in the DA group in comparison to the D group suggests that the mushroom exerts an effect on the metabolic control of urea. In contrast, no significant differences among groups were found regarding creatinine levels.

The analysis of transferrin and iron was another important aspect of the present study. Transferrin, which is a glycoprotein, is the main protein in the blood that carries iron.
throughout the body. Iron is an important element to many functions of the body, but its main role is to be the building block for hemoglobin, which helps red blood cells bind to oxygen for delivery to tissues. Chronic or inflammatory diseases, such as diabetes, result in low transferrin and iron, which is a sign that the liver cells are damaged and the transport of these ions through beta globulin cells in the blood stream is impaired [43, 44]. In the present study, decreased transferrin and iron were found in the animals of the D group, indicating chronic inflammation. However, the incorporation of the mushroom into the food in the DA group led to a statistically significant increase in both transferrin and iron. No significant differences were found between the C and CA groups.

Alkaline phosphatase is distributed in all tissues. An increase in this enzyme in the blood indicates the occurrence of cytolysis and the release of liver cell contents due to damage or necrosis. The increase found in the D group is consistent with results obtained in diabetic patients [45–48]. GPT is found predominantly in the liver at a particular concentration. When liver tissue is damaged and the parenchyma is affected, a greater quantity of this enzyme is released into the blood stream. Thus, the increase in this enzyme found in the D group indicates liver cell damage. However, reductions in both of these enzymes were found in the DA group.

Thinking in all these data together an intraperitoneal injection of the chemotherapy agent streptozotocin damages the pancreas and results in the loss of β-cells and degeneration of the islets. This may be through a pathomechanism that involves a local inflammatory response in the pancreas triggered by streptozotocin-induced cell death. These animals are expected to be sick and hence would not grow well despite eating well. The kidneys and liver could also be affected by this insult and hence the increase of urea and liver enzymes and the decrease of transferrin and iron levels. Extracts from A. sylvaticus may have a systemic immunomodulatory effect (as opposed to a pancreatic stimulatory effect) that attenuates this inflammatory response, hence resulting in a lesser degree of β-cells loss and islet degeneration. The surviving islets and cells would have to compensate and hence become hyperplastic; this is consistent with our findings. It will also lead to less manifestations of diabetic status, as shown in the data, and some degree of kidney and liver protection and hence improved, respectively, urea, enzymes, and transferrin plasma levels.

In conclusion, the present findings demonstrate that Agaricus sylvaticus is potentially beneficial in the control of type 1 diabetes by reducing blood glucose, cholesterol, and triglyceride levels, increasing HDL cholesterol and regulating GPT, alkaline phosphatase, iron, transferrin, and urea levels. It may improve the pancreas function increasing the number of the cells in the islets of Langerhans which ameliorate the symptoms of the disease toward the homeostasis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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