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Pancreas Protective Effect of Button Mushroom *Agaricus bisporus* (J.E. Lange) Imbach (Agaricomycetidae) Extract on Rats with Streptozotocin-Induced Dia betes

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ABSTRACT: In the present study we describe the effects of hot water extract of the culinary-medicinal button mushroom, *Agaricus bisporus*, on the symptoms of streptozotocin-induced diabetes in Sprague Dawley rats. *A. bisporus* extract at the doses of 0, 100, 200, and 400 mg/kg body weight (bw) per day were orally applied to streptozotocin-induced diabetic rats for a period of 7 days after the onset of the diabetes. Food and water intake and body weight were recorded daily. Upon sacrifice, histological studies were performed on pancreas tissues, and biochemical parameters such as glucose, insulin superoxide dismutase, malondialdehyde and catalase were measured of all experimental groups. The serum glucose levels significantly decreased after oral administration at the dose of 400 mg/kg bw per day by 29.68% with *A. bisporus* extract. Furthermore, the serum insulin levels in the streptozotocin-induced diabetic rats were increased to 78.50% at the extract dose of 400 mg/kg bw per day. Also, catalase activities and malondialdehyde levels decreased to values slightly above the normal animal control. The most obvious and surprising change was, however, the increase in cellularity of the Langerhans islets of the pancreas and their apparent repopulation with beta cells. We conclude that the oral application of high doses of *A. bisporus* extract may result in decreased severity of streptozotocin-induced diabetes in rat.

KEY WORDS: medicinal mushrooms, *Agaricus bisporus*, diabetes, hot water extract, hypoglycemic effect, pancreas histology, streptozotocin

ABBREVIATIONS: ABE: *Agaricus bisporus* extract; **bw**: body weight; **CAT**: catalase; **H & E**: hematoxylin and eosin; **MDA**: malondialdehyde; **NBT**: nitrobluetetrazolium; **SOD**: superoxide dismutase; **STZ**: streptozotocin

I. INTRODUCTION

Diabetes mellitus is a serious, complex chronic disorder, which is rapidly increasing in the Western world in numbers of people affected. Fleming, Schellevis and Van Kasteren[1] reported a prevalence of diabetes in eight European countries for people aged 45–64 years ranging from 33.3 to 58.1 per 1000, and from 21 to 53 per 1000, in males and females, respectively. According to the World Health Organization, approximately 171 million people worldwide were suffering from diabetes in 2000. This number is projected to double by 2030.[2]

Diabetes mellitus is characterized by hyperglycemia dependent on its absolute (type I) or relative (type II) lack of insulin.[3] The basis of type 1 diabetes is the destruction of the β cells of the pancreatic Langerhans islets. Pancreatic β-cells are reportedly vulnerable to oxidative stress, which may induce β-cell

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apoptosis and a decrease in β-cell mass.[4] This decrease results in the dysfunction of insulin secretion, and leads to the onset of diabetes. The susceptibility of β-cells to oxidative stress may be related to their possessing relatively impaired antioxidant defense mechanisms such as Cu/Zn superoxide dismutase (SOD), Mn SOD, catalase (CAT), and glutathione peroxidase.[4] It has been shown that patients with diabetes mellitus have increased oxidative stress and impaired antioxidant defense systems, which appear to contribute to the initiation and progression of diabetes-associated complications.[5] Complications include hypoglycemia and hyperglycemia, increased risk of infections, microvascular complications (eg, retinopathy, nephropathy), neuropathic complications, blindness, nontraumatic lower-extremity amputation and end-stage renal disease. Antioxidant nutritional supplements or antioxidant-containing foods may be used to prevent oxidative stress-mediated damage. Phenolic compounds, carotenoids, tocopherol and ascorbic acid are the most important antioxidant molecules contained in foods, especially in vegetables. Mushrooms are rich sources of antioxidant compounds, especially of phenolic compounds and ergothioneine.[6–8]

Hot water extracts of *Ganoderma lucidum*[9] and of *Agaricus brasiliensis*,[10] which contain β-glucans, small amounts of protein and triterpenoids were found to have anti-diabetic effects when given to rats with diabetes induced by streptozotocin (STZ).

In the present study we describe the effects of orally administered hot water extracts of the common white button mushroom *Agaricus bisporus* (J.E. Lange) Imbach (Agaricaceae, Agaricomycetideae) on symptoms of STZ-induced diabetes in rats including blood glucose and oxidative stress parameters, and histological effects on pancreas structure. In the late eighties of last century it was already found that *A. bisporus* could reduce symptoms of STZ-induced diabetes in mice.[11] To our knowledge, our present study is the first to show therapeutic effects of orally given *A. bisporus* hot water extract in diabetic rats based on biochemical and histological parameters.

II. MATERIALS AND METHODS

A. Biological Materials

Extracts were made of fruiting bodies of *Agaricus bisporus* Sylvan A15, which is a commercial substrain of Horst U1. The pure culture of *A. bisporus* Horst U1 was deposited at ATCC under number 62462. *A. bisporus* extract (ABE) was prepared from homogenized fresh white button mushrooms by prolonged hot water extraction. The resulting mash was band pressed and residual solid components were removed by repeated centrifugation (2000 g) and filtration. Finally, the extract was concentrated to 35 °Brix by water removal through ultrafiltration. The extract was then treated with 2 volumes of ethanol 96% and the resulting precipitate was collected, dissolved in water and reprecipitated by 2 volumes of ethanol. The final precipitate was dried and stored for later use. Its composition was determined as published before.[12] Typical extracts consisted of approximately 95% polysaccharide, 1% protein and 4.0% polyphenolic compounds.[13]

B. Chemicals and Reagents

Streptozotocin (STZ), nitro blue tetrazolium (NBT), and thiobarbituric acid and hydrogen peroxide were obtained from Sigma, St. Louis, MO (USA).

C. Animals

Male Sprague Dawley rats (200-230 g) were procured from TICAM (Medical and Surgical Experimental Research Centre, Eskisehir

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Osmangazi University) and housed in polycarbonate cages in an air-conditioned room (22 ± 2°C) with a 12 h light: 12 h dark cycle (07:00 h on, 19:00 h off). Standard rat feed and water were provided *ad libitum*. The rats were allowed to acclimatize to the laboratory environment for 7 days before the start of the experiment.

All procedures were conducted in conformity with the Institutional Ethical Committee for Animal Care and Use at Eskisehir Osmangazi University (protocol number 87/2008) and the international guidelines on the ethical use of animals (NIH publications No. 80-23).

D. Induction of Diabetes in Rats

Diabetes was induced following overnight fasting of the rats by an intramuscular injection of STZ in a single dose of 50 mg/kg. STZ was dissolved in a freshly prepared 0.01 M citrate buffer (pH 4.5) while the control rats were injected with buffer alone. Five days after STZ administration, the diabetic state was confirmed by the positive response of glucose in urine (using strips [Arkray, Kyoto, Japan]).

E. Experimental Design

The rats were randomly divided into five groups (n = 8 per group). Group I: control animals, Group II: STZ-diabetic control animals, Group III: STZ-diabetic animals given ABE 100 mg/kg bw per day, Group IV STZ-diabetic animals given ABE 200 mg/kg bw per day, and Group V: STZ-diabetic animals given ABE 400 mg/kg bw per day. ABE was dissolved in isotonic sodium chloride and given by gavage daily for seven days. The saline solution was given to groups I and II in the same way. The schedule of the experimental period is presented in Table 1.

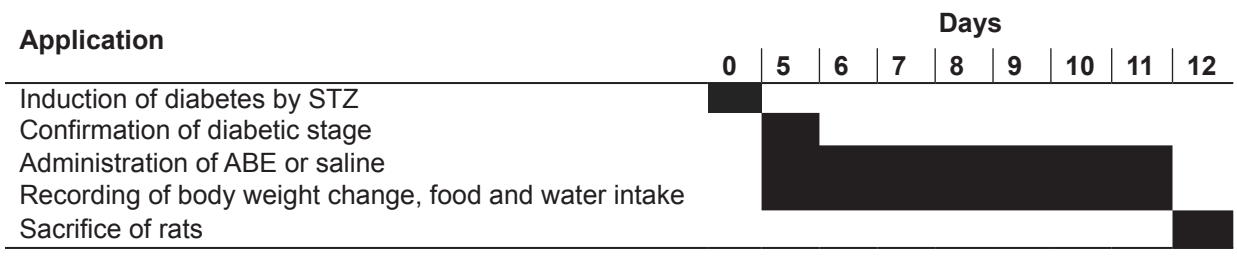
During the experimental period, body weight changes, and food and water intake were recorded daily. At the end of the experimental period, all animals were fasted for 9 hours and sacrificed by ether anesthesia. Pancreas tissues were removed and rinsed with cold saline and then used for determination of weight, histological evaluation, and measurement of enzyme activities.

F. Biochemical Analysis

In this study, biochemical investigations were made in serum and pancreas tissue. Blood samples from rats were collected in polystyrene tubes without anticoagulant. Pancreatic tissue malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT) were measured in samples stored at –80° C.

Serum was separated by centrifugation at 1,600 g at 4°C for 15 minutes using a cooling centrifuge (Hermle ZK510, Germany) and analyzed for serum glucose and insulin levels. The serum glucose levels were immediately measured with a commercial kit (Biolabo, France) using an auto analyzer (Crony Instruments, Airone 200-RA, Italy). The serum insulin level of each blood sample was measured by an enzyme-linked immunosor-

TABLE 1. Schedule of the Experimental Period



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bent assay using a commercial kit (ultra sensitive rat insulin enzyme-linked immunosorbent assay; catalog number 10-1197-01; Mercodia, Uppsala, Sweden), based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. Serum glucose and insulin levels were expressed as mg/dL and μg/L, respectively.

1. Malondialdehyde Measurement

Pancreatic tissue was homogenized in 0.15 M KCl buffer (1/10) and the homogenized material was centrifuged at 1500 g for 10 min. The supernatant fraction was removed for the determination of MDA levels. All tissue homogenates were stored at –80°C. Lipid peroxidation was determined by the measurement of the malondialdehyde - TBA assay, according to Ohkawa et al.[14] Absorbances were measured at 532 nm. MDA levels were expressed as nmol/mg protein.

2. Superoxide Dismutase and Catalase Activities

For the assay of SOD and CAT activities, pancreatic tissues were homogenized in 50 mmol/L phosphate buffer (pH 7.8). The homogenate was centrifuged at 1500 g for 10 min. The supernatant was used for the assay in a 1:10 dilution. All tissue homogenates were stored at –80°C.

SOD activity was measured according to Beaucham and Fridovich's method as modified by Winterbourn et al.[15] The method measures the inhibitory effect of SOD on the reaction in which superoxide anion reduces NBT. Absorbances were measured at 560 nm. Tissue SOD activities were expressed as U/mg protein.

The activity of CAT in tissue was determined according to Beutler's method,[16] based on decomposition of H_2O_2, as observed by the direct decrease of absorbance at 230 nm. Tissue CAT activities were expressed as U/mg protein.

Protein levels of pancreatic tissue were determined by the biuret method.[17]

G. Histological Evaluation

The pancreas was removed from the animals immediately after sacrifice and rinsed in ice-cold saline. Tissues were fixed in 10% neutral formalin for at least 24 hours and processed for light microscopy. Each piece of pancreas embedded in paraffin blocs was sectioned at 5 μm in length before applying on slides for histological staining. Slides were stained with hematoxylin and eosin for general observation, and with modified Gomori's Aldehyde Fuchsin stain (0.5% basic fuchsin solution in 70% alcohol, Lugol's iodine, 0.5% sodium bisulfite in distilled water, 0.25% metanil yellow solution in distilled water) for intense staining of beta cells in pancreatic islets. Micrographs of slides were taken by an Olympus (Tokyo, Japan) BX 50 microscope with an adapted Olympus DP70 digital camera. Each digital micrograph of the Langerhans islets was studied for changes of the surface area of the Langerhans islets and of the beta cells respectively and of the number of cells using an image analyzing program (Soft Imaging System LS version 5.0).

H. Statistical Analysis

All data were analyzed using the SPSS (Chicago, IL, USA) Statistical Package (version 12.0) statistical software with $p < 0.05$ values considered as statistically significant. Comparisons between the groups were assessed by one-way analysis of variance (ANOVA) with Tukey's multiple range post hoc tests. Results were expressed as mean±SD.

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III. RESULTS AND DISCUSSION

A. Body Weight, Daily Food and Water Intake, and Pancreas Weight Values

In this study, there was an increased daily food intake found in the diabetic and ABE treated groups depending on the ABE treatment of the diabetic animals. Table 2 depicts the levels of initial and final body weight, daily food intake, daily water intake, and pancreas weight in control and experimental groups of rats. There was no significant difference between initial and final body weight in the groups of diabetic rats with or without ABE (100, 200 and 400 mg/kg bw per day) treatment. This was mainly due to the large deviations from the average weights within the groups. Body weight loss and increased food intake are a general phenomenon in STZ-induced diabetic rats. But this situation may recover during the treatment. The present study showed that the diabetic rats' body weight slightly increased after oral treatment by ABE. These results confirm those by Kim et al.[18] in their study of the effects of oral administration of extracellularly produced polysaccharides of five different medicinal mushrooms in diabetic rats.

The water intakes of the rats significantly decreased, mostly at the 400 mg/kg bw per day ABE treated diabetic group (37.44±2.13 mL/100g bw) compared to diabetic control group (43.08±2.26 mL/100g bw) ($p < 0.05$). A similar correlation was also seen between pancreas weight values. Significant increases were recorded for pancreas weights of the groups at doses of 200 and 400 mg/kg bw per day compared to the diabetic control group (Table 2).

TABLE 2. Body Weight, Daily Food and Water Intake, and Pancreas Weight Values in Control and Experimental Groups of Rats

Experimental groups*	Initial body weight (g)	Final body weight (g)	Daily food intake (g/100g bw**)	Daily water intake (ml/100g bw)	Pancreas weight (g/kg bw)
NC	255.50±18.58	278.19±15.28	9.10±0.52 a	12.37±1.33 a	29.00±2.26 a
DC	218.40±30.11	198.40±29.30	14.73±2.25 b	43.08±2.26 b	20.62±1.18 b
100	247.75±8.77	238.75±9.25	13.20±2.25 b	44.05±3.06 b	22.75±1.58 bc
200	243.75±9.58	229.00±10.84	15.11±1.40 b	41.77±1.72 b	23.87±1.80 c
400	249.75±22.12	238.50±18.03	14.20±1.47 b	37.44±2.13 c	26.75±3.32 d

* For details, see Materials and Methods section. **bw: Body weight. Mean±SD, one-way ANOVA. Values in each column followed by the same letter do not differ significantly ($p < 0.05$).

B. Change of Serum Glucose and Serum Insulin Level in Diabetic Animals After ABE Administration

Diabetes is a metabolic disorder characterized by hyperglycemia resulting from a deficit in β-cell mass and insulin secretion or action, or both. Hyperglycemia is blamed for the complications of diabetes because an elevated glucose concentration directly injures cells and induces lipid peroxidation. As shown in Table 3, STZ treatment caused a significant increase of serum glucose (380.70%) when compared to the non-diabetic control group ($p < 0.05$). At the same time, the insulin level decreased to 31.6 % of the control value. In

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this dose-dependent study, treatment of the diseased animals with ABE at the doses of 100, 200 and 400 mg/kg bw per day showed a reduced level of serum glucose (8.29%, 22.59% and 29.68%, respectively). Especially, ABE at the doses of 200 and 400 mg/kg bw per day led to a significant decrease ($p < 0.05$) in serum glucose levels.

ABE treated experimental groups (100, 200 and 400 mg/kg bw per day) represented a dose-dependent increase in the serum insulin levels (16.66%, 39.47% and 78.50%, respectively). Statistically, a remarkable increase was observed in the serum insulin values for the ABE groups at the doses of 200 and 400 mg/kg bw per day ($p < 0.05$). Although administration of ABE at the doses of 200 and 400 mg/kg bw per day significantly reduced the serum glucose levels and increased the serum insulin levels, the same value as that of the normal control was not achieved after the 7 days of treatment (Table 3).

TABLE 3. Serum Glucose and Insulin Levels in Control and Experimental Groups of Rats

Experimental groups*	Serum Glucose levels (mg/dL)	Serum insulin levels (µg/L)
NC	141.46±26.49 a	7.20±0.80 a
DC	538.53±21.54 b	2.28±0.42 b
100	493.84±59.61 b	2.66±0.22 b
200	416.84±62.44 c	3.18±0.77 bc
400	378.65±33.25 c	4.07±1.19 c

* For details, see Materials and Methods section. Mean±SD, one-way ANOVA. Values in each column followed by the same letter do not differ significantly ($p < 0.05$).

C. Changes in MDA, SOD, CAT

In our study, MDA levels were increased significantly in diabetic animals as compared to the non-diabetic control group ($p < 0.05$). But, MDA levels in diabetic animals were significantly decreased by the treatment of 200 and 400 mg/kg bw per day ABE ($p < 0.05$). At an MDA level of 400 mg/kg bw per day the ABE treatment group almost reached the level of the normal control animals ($p < 0.05$). In the diabetic control group, SOD levels were found to be higher than those of the non-diabetic control group ($p < 0.05$). The ABE groups at 100 and 200 mg doses were significantly lower in SOD activity than the diabetic animals ($p < 0.05$). In contrast, the SOD activity in the 400 mg group was higher than in the non-diabetic control animals and the other treated groups ($p < 0.05$). The CAT activities in all treatment groups were decreased slightly when compared to diabetic control animals. But there was no statistically significant difference ($p < 0.05$). CAT activities in all treatment groups were also found to be higher than in the NC group ($p < 0.05$) and experimental groups did not reach the value of healthy control animals (Table 4).

Our biochemical findings show that 200 and 400 mg/kg bw per day doses of ABE may have hypoglycemic effects. How does this relate to the role that free radicals play in diabetes? There are several proposed mechanisms of free radical production in diabetes:

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glycoxidation, change in intracellular NADH/NAD ratio (hyperglycemic pseudohypoxia), and effects on prostaglandin biosynthesis. Since expression of antioxidant enzymes may be induced at the transcriptional level by oxidative stress, it is possible that the metabolic changes accompanying diabetes may induce these enzymes.[19] Wohaieb and Godin[20] observed a decrease of SOD activity in the liver and kidney and an increase in the pancreas of STZ treated diabetic rats. They proposed that increased enzyme activity might be an adaptive response in the otherwise SOD-poor pancreas, while the reduction of SOD activity in liver and kidney might be due to the direct damaging effect of free radicals on the enzyme. Several reports suggest that heart and pancreas tissues show increased CAT activity in the diabetic state.[20]

In our study, ABE seems to prevent diabetes mediated peroxidative injury as suggested by the decreased MDA levels in the pancreatic tissue of STZ-induced diabetic rats. Besides, CAT activities were decreased slightly in all doses. SOD activities in the 100 and 200 mg/kg bw per day ABE groups were significantly decreased as compared to the diabetic control group. Therefore, these findings suggest that ABE may be useful against diabetes induced oxidative stress. To our surprise, however, the SOD activity of the group treated with ABE at the dose of 400 mg/kg bw per day was significantly increased. The finding of increased SOD activity in the 400 mg ABE treatment group is of sufficient interest to support new investigations. Possible toxicity of this dose has not been observed.

TABLE 4. Malondialdehyde, Superoxide Dismutase and Catalase Levels in Control and Experimental Groups of Rats

Experimental groups*	MDA (nmol/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)
NC	2.24 ± 0.69 a	6.53 ± 1.56 a	0.83 ± 0.41 a
DC	4.04 ± 0.72 b	8.56 ± 1.72 b	1.54 ± 0.71 b
100	3.42 ± 1.29 b	5.83 ± 1.81 a	1.01 ± 0.51 ab
200	3.18 ± 0.77 ab	6.13 ± 2.93 a	1.09 ± 0. 68 ab
400	2.20 ± 0.63 a	9.96 ± 1.38 b	1.11 ± 0.61 ab

* For details, see Materials and Methods section. Mean±SD, one-way ANOVA, values in each column followed by the same letter do not differ significantly ($p < 0.05$).

D. Histological evaluation

Streptozotocin (STZ), a monofunctional nitrosourea derivative, is one of the most commonly used substances to induce diabetes in experimental animals. The diabetogenic capacity of STZ may depend on its ability to damage ß-cells and induce oxidative stress. The results of histological studies are summarized in Table 5. In the diabetic group, three of the studied parameters (area of Langerhans islets, area of beta cells in the islets, number

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of total cells in the islets) were significantly lower than in non-diabetic control animals ($p < 0.05$). All parameters in the ABE treated animals at the doses of 100 mg/kg bw per day were changed slightly compared to diabetic control animals. But, ABE at doses of 200 and 400 mg/kg bw per day led to an increase in all histological parameters compared to the diabetic control group (Table 5).

In the non-diabetic control group, all cells in the islets of Langerhans were strongly stained with hematoxylin and eosin. Dark brown-violet colored nucleated cells appeared mainly homogenously scattered (Fig. 1A). Also, aldehyde fuchsin staining, which is specific for compounds such as insulin in beta cells, strongly stained part of the islets dark violet-blue (Fig. 1F). It should be taken into account that this stain has also affinity to compounds that are structurally related to insulin.

There were remarkable histological differences between the diabetic and nondiabetic control groups. In the diabetic control group, the average area and number of beta cells in the islets of Langerhans of the diabetic control group were dramatically decreased (Fig. 1A, 1B, 1F, 1G; Table 5). The area of beta cells of the pancreatic islets was decreased by 74%, presumably resulting in severe functional damage. The stained histological slides of the 100 mg ABE treated group were quite similar to the diabetic control group (Fig. 1C, 1H). But the slides of the 200 and 400 mg ABE treated groups showed that the number of beta cells and the area of the islets of Langerhans had clearly increased (Fig. 1D, 1E, 1I, 1J). Especially, the insulin levels in the 200 mg ABE group seemed increased (Fig. 1D, 1I) in the aldehyde fuchsin stained slides. In our study we showed that the surface area of the Langerhans islets and of beta cells, and the number of total cells of STZ-induced diabetic rats clearly increased related to the doses of ABE that were applied to the animals. The pancreas protective effect of ABE extract was supported by biochemical data.

Our study leads to the following conclusions. STZ-induced diabetes in rats rapidly leads to hyperglycemia and to oxidative stress. In agreement with previous findings by others,[18,19] our results showed that SOD and CAT activities were increased in the diabetic control group as compared to the normal control group ($p < 0.05$). Increased enzyme activities might be an adaptive re-

TABLE 5. Histological Values of Pancreatic Tissues in Control and Experimental Groups of Rats

Experimental groups*	Area of Langerhans Islets (nm2)	Area of Beta Cells in Langerhans Islets (nm2)	Number of Total Cells in Langerhans Islets
NC	33762.8 ± 2183 a	28972.78 ± 1689 a	127.40 ± 21.8 a
DC	15852.95 ± 798 b	7666.43 ± 489 b	74.00 ± 25.5 b
100	15980.2 ± 983 b	13320.98 ± 866 b	69.25 ± 34.7 b
200	22307.76 ± 1864 ab	18802.23 ± 1263 ab	83.80 ± 41.06 ab
400	28354.96 ± 1163 ab	19199.28 ± 1081 ab	86.00 ± 41.93 ab

* For details, see Materials and Methods section. Mean±SD, one-way ANOVA, Values in each column followed by the same letter do not differ significantly ($p < 0.05$).

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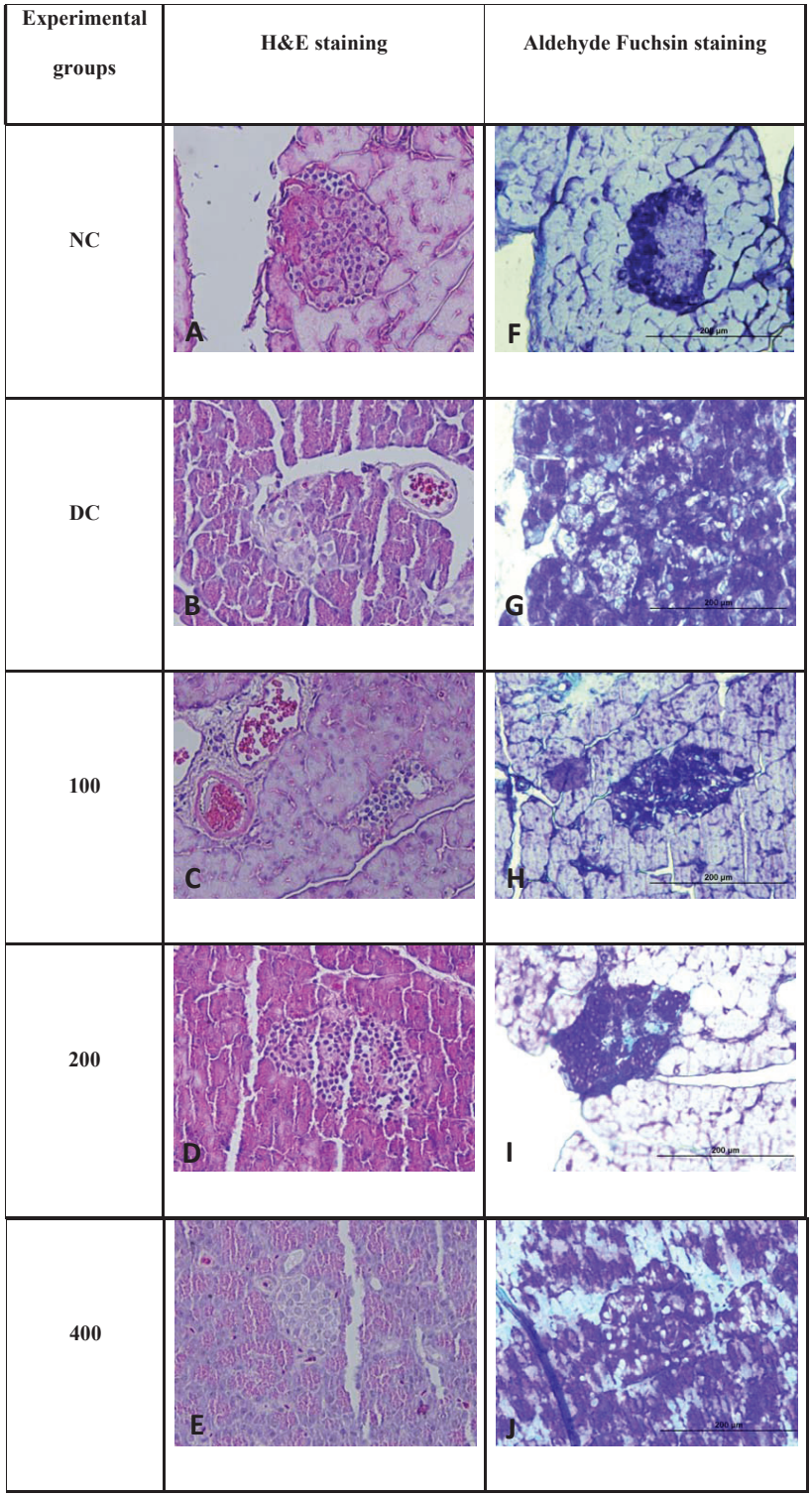

FIGURE 1. Histological micrographs stained with H&E (A-E) and modified aldehyde fuchsin (F-J) of pancreatic tissues in control and experimental groups of rats (scale bar: 200 µm).

Histological micrographs stained with H&E (A-E) and modified Aldehyde Fuchsin (F-J) of pancreatic tissues in control and experimental groups of rats (Scale bar: 200 mm.).

sponse against oxidative stress in diabetes mellitus. In this study, we demonstrated that MDA levels, a lipid peroxidation end-product and a marker of oxidative stress, were elevated in the diabetic control group. Increased MDA levels are caused by free radical-induced lipid peroxidation in diabetes mellitus as described in the literature.[21]

Beta cells are particularly sensitive to free radicals because they are low in antioxidant enzymes such as peroxidase, CAT and SOD.[22] As mushrooms were known to harbor considerable concentrations of antioxidants,[7] we looked into the effects of extracts of *A. bisporus*, the white button mushroom on STZ-induced diabetes. *A. bisporus c*ontains high amounts of the antioxidant amino acid ergothioneine,[7,23] which in combination with the receptor/transporter OCTN1, is able to pass the cell membrane and to protect the cells from free radical damage.[24] Although studies looking at the use of potential antioxidants to reduce oxidant stress have not been encouraging,[25] mushroom ergothioneine-OCTN1 could form an exception because this complex enters the cell to maintain redox balance against oxidant conditions instead of remaining in the pro-oxidant plasma environment and becoming rapidly degraded. It is tempting to suggest that beta cells just as skin cells contain a transporter that specifically allows ergothioneine to be internalized in the cells and exert its antioxidative action.[24]

In conclusion, based on the pancreas protective effect as confirmed by functional and morphological repair and the increase of antioxidative defense enzyme activity, we assume that *A. bisporus* hot water extract may be useful as an oral agent in the treatment of diabetes.

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