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Preliminary Study Results of Native Mongolian *Inonotus Obliquus* Extracts in Alloxan-Induced Diabetic Rats

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Objectives: We aimed to investigate the morphological changes and clinical chemistry panel of alloxan-induced diabetic rats for the treatment groups of ethanol or aqueous extracts of Mongolian natural *Inonotus obliquus*. **Methods:** Wistar albino rats (n = 80) were randomly assigned into four groups: 1. Healthy control group, 2. Untreated diabetic group, 3. Diabetic group treated with *Inonotus obliquus* (500 mg/kg per day) water extract, 4. Diabetic group with *Inonotus obliquus* (500 mg/kg per day) ethanol extract. Diabetes was induced by alloxan 150 mg/kg (single intraperitoneal injection) in the diabetic groups. **Results:** Both water and ethanol extract of *Inonotus obliquus* reduced the blood glucose level of diabetes at 48 h after treatment. Histologic and morphology examinations showed that water extracts of *Inonotus obliquus* alleviated the damage to pancreas tissues in alloxan diabetic rats. **Conclusions:** We postulate that simple ethanol and water extracts of natural Mongolian *Inonotus obliquus* have an antihyperglycemic effect that enhances islet cell function in alloxan-induced diabetic rats. The extracts of Mongolian natural *Inonotus obliquus* are easily producible at low cost and should be further explored as a treatment for diabetes.

Keywords: *Inonotus obliquus*, Diabetes, Alloxan, Langerhans Islet, Traditional Medicine

Introduction

Diabetes mellitus describes a group of metabolic disorders characterized by increased blood glucose concentration, decreased insulin and insulin tolerance [1]. According to the American Diabetes Association, the medical costs for a person with diabetes are twice that of people without diabetes. Of that amount, over 60% is attributed to the direct treatment of diabetes [2]. In Mongolia, 50-75% of diabetic patients who required insulin did not have access to it due to high medication costs [3]. The medication costs of blood glucose-lowering drugs, including insulin and others, rose by 45% between 2012 and 2017, and the total treatment cost for patients with diabetes mellitus (type 1 and 2) has increased significantly in recent years [4]. Numerous extracts from medicinal mushrooms are known to attenuate diabetes in experimental animal design studies and have been shown to delay diabetic complications [5]. Current diabetes mellitus medications have various limitations, such as adverse effects, limited efficacy, and high rates of secondary failure. Therefore, the current complementary and alternative medicine research prioritizes the search for new glucose-lowering agents that are affordable, and with fewer adverse effects, across the world [6, 7].

Inonotus Obliquus (IO) is one of the widely known medicinal mushrooms and is commonly used in traditional medicine (especially by Khanty people) to treat various health conditions. It is widely known as the Chaga mushroom, belonging to the Hymenochaetaceae family of Basidiomycetes, a naturally parasitic fungus growing on birch trees. Since the 16th century, IO has been used in East Europe, Central Asia, particularly the Siberian regions of Russia and Mongolia, for traditional medicine for diabetes [8]. Previous phytochemical analyses of IO reported the presence of active compounds, including polyphenol, triterpenoids and steroids [9-12]. Moreover, these substances potentially have anticancer, antioxidant, antiviral, anti-inflammatory and analgesic effects [13-18]. In the last decade, researchers focused on the antidiabetic effect of IO. Fermented or submerged culture of IO reduced the levels of free fatty acids, cholesterol, triglyceride and low-density lipoprotein, with a notable enhancement of high-density lipoprotein, insulin levels and hepatic glycogen contents of the liver in diabetic mice and rat models [19-21]. In addition, polysaccharides from IO also have potential antitumor, antioxidant and antihyperlipidemic effects [22-24].

These traditional medicines have great potential in developing countries and rural areas, where most individuals with diabetes have limited resourses and access to medications [25]. Moreover, the recognition of traditional medicines is rising worldwide due to lower toxicity levels and their natural origins [26]. IO has been widely used in Mongolian traditional medicine to treat gastric disorders as Chaga mushroom tea and water extract form a natural sclerotome of IO. However, the aqueous and ethanol extracts from natural Mongolian IO have not been explored, which is immediately noted among the people. Therefore, we examined the antihyperglycemic, antihyperlipidemic and some regenerative effects on Langerhans islet cells in an alloxan-induced diabetic rat model using simple aqueous and ethanol extracts from natural Mongolian IO that are widely used by the Mongolian general population.

Materials and Methods

Preparation of aqueous and ethanol extract

We used naturally abundant Mongolian *Inonotus Obliquus* that was initially collected and treated by the Mongolian Traditional Medicine and Herb Factory, which ensured species identification. Fifty-grams of powdered Inonotus Obliquus was added into a round bottom flask followed by 500 ml of distilled water (1:10), and the extract was produced by reflux at 100°C for 2 h. The mixture was continuously stirred during the reflux for homogeneous mixing and heat distribution. After the reflux extraction, the sample was cooled to room temperature. The extract was diluted with distilled water until the initial desired concentration was achieved.

The ethanol extract was prepared with a 1:10 ratio. The powdered *Inonotus Obliquus* was extracted with 40% ethanol (50g of powder/500 ml 40% ethanol) by heating at 70^{0}C for 6 h. Both extracts were stored at 4^{0}C and initial extracts were filtered by 0.22 μm pore syringe filters.

Reagents

Alloxan monohydrate (Sigma-Aldrich Chemical Co., USA) was used in the experiment. Chemiluminescent reagent kits (Human Biochemica and Diagnostica GmbH, Wiesbaden, Germany) were used to measure the levels of serum total cholesterol (TC), triacylglycerol (TG), low-density lipoprotein (LDL), high-density lipoprotein (HDL). Other remaining widely used chemicals and reagents were of analytical grade.

Inducing diabetes in rats using alloxan

Eighty Wistar albino rats (180 ± 20 gm) were supplied by the Traditional Medical Science and Production Corporation of Mongolia. The rats were housed at 20 ± 2^{0}C temperature with an alternating 12 hours light and 12 hours dark cycle with a standard feeding. Diabetes was induced in rats in all except those serving as healthy negative controls. The rats fasted for 16 hours before intraperitoneal injection of alloxan monohydrate 150 mg/kg once. We confirmed the induction of diabetes with the criteria of blood glucose level ≥ 11.1 mmol/l after 48 hours of alloxan injection. Peripheral blood glucose level was measured on blood collected from the rat tail vein using a blood glucose meter (Accu-Chek Active, Roche Diabetes Care, Inc., USA). All experiments proceeded after confirming diabetes in the animals.

Groups and treatments

We allocated the rats randomly into 4 groups. Group I was the healthy negative control group (n = 20) that received only distilled water orally, and the remaining three groups were alloxan-induced diabetic rats. Group II was treated with orally administered distilled water as well (n = 20). Group III received the water extract of IO (WEIO) at a dose of 500 mg/kg body weight (n = 20). Meanwhile, the Group IV rats received ethanol extract of IO (EEIO) at a dose of 500 mg/kg body weight (n = 20). The extracts were administered via oral gavage three times per day in a total fluid volume of 1 to 1.25 ml. Ten of the rats in each of the 4 groups were slaughtered for blood and tissue sampling at 24h, and the remaining ten at 48h after the administration of the extracts.

Biochemical analysis

A 5 ml blood sample was collected from the jugular vein. After centrifuging at 3000 x g for 10 min at 4^{0}C, the serum was collected for determination of blood glucose levels, TC, TG, LDL and HDL levels using commercial kits.

Histopathological examination

The rats were euthanized under sodium pentobarbitone anesthesia according to the guidelines for euthanasia in the Guide for the Care and Use of Laboratory Animals at 24 and 48 hours after IO administration. The pancreas was harvested, and a 0.5 x 0.5 cm sample was fixed in 10% neutral buffered formalin (pH = 7.4) for 24 hours. We washed the sample afterward for 24 hours, followed by dehydration by soaking the tissue in increasing concentrations of ethyl alcohol in an aqueous solution. The dehydrated tissue was then cleared in xylene and embedded in paraffin. Slides were made 5-µm-thick, and cut into sections. We stained the pancreas tissue with hematoxylin and eosin, and morphologic analyses were performed using light microscopy (Olympus BX53, Olympus Life Science Solutions, USA).

Statistical analysis

The data for each group were expressed as mean ± standard deviation (n = 10 per group). Because of the small sample sizes, examination for differences among the four groups was conducted using the Kruskal-Wallis test with a critical p-value of $p < 0.05$. Multiple post-hoc comparisons were performed using the Wilcoxon sign rank test. The critical p-value for the post-hoc tests was adjusted using the Bonferroni method. Since there were 4 groups, there were 6 possible pairwise comparisons. Consequently, the critical p-value for the post-hoc tests was $p < 0.05/6 = 0.0083$. All analyses were performed using SPSS 23.0.

Ethical statements

The experimental protocols were designed under ethical guidelines and the study protocol was approved by the Biomedical Research Ethical Review Board, Mongolian National University of Medical Sciences, under permission No2016/3-2016-22. IO is a naturally abundant species in Mongolia with no restrictions on consumer usage.

Results

Blood glucose level reduction in extract-treated groups

The effects of WEIO and EEIO on blood glucose level in alloxan-induced diabetic rats are presented in Tables 1 and 2. The blood glucose level in the diabetic group increased after 24 hours and 48 hours of alloxan injection. We did not observe significant differences between WEIO and EEIO treatment groups and the diabetic group 24 hours after treatment. However, at 48 hours after treatment, the blood glucose level was significantly lower in both the WEIO and EEIO-treated groups (8.8 ± 2.23, 9.88 ± 1.21 mmol/l), compared with the diabetic group (17.07 ± 1.6 mmol/l, $p < 0.05$).

Extract effects on levels of serum lipids and lipoprotein

The effect of WEIO and EEIO on serum lipids and lipoprotein in alloxan-induced diabetic rats are summarized in Tables 1 and 2. The mean serum TC, TG, and LDL levels in the diabetic group increased at 24 hours and 48 hours following alloxan injection. Treatment of WEIO and EEIO tended to decrease the mean levels of TC, TG, LDL, and to increase HDL, but these trends were not statistically significant.

Table 1. Effect of WEIO and EEIO treatment on serum plasma glucose, lipids and lipoprotein levels after 24 hours treatment.

Variables	Control (n = 10)	Diabetic (n = 10)	WEIO treated (n = 10)	EEIO treated (n = 10)	*p-value
Glucose (mmol/l)	5.73 ± 0.29^{ab}	8.63 ± 2.59^{b}	7.27 ± 1.68^{a}	8.01 ± 3.06	0.003
TC (mmol/l)	3.11 ± 0.79^{cd}	4.00 ± 0.47^{d}	3.93 ± 0.7^{c}	3.78 ± 0.53	0.030
TG (mmol/l)	1.65 ± 0.67^{ef}	2.75 ± 0.71^{f}	2.57 ± 0.46^{e}	2.62 ± 0.74	0.018
HDL (mmol/l)	1.69 ± 0.16	1.96 ± 0.49	1.95 ± 0.45	1.81 ± 0.31	0.449
LDL (mmol/l)	1.26 ± 0.37^{gh}	1.71 ± 0.11^{h}	1.51 ± 0.21^{g}	1.66 ± 0.09	0.001

EEIO - ethanol extract of Inonotus Obliquus, WEIO - water extract of Inonotus Obliquus, TC - total cholesterol, TG – triglyceride, LDL - low-density lipoprotein, HDL - high-density lipoprotein. *Kruskal-Wallis test with $p < 0.05$, ^{abcdefghjkl}pairwise comparison using Wilcoxon test with $p < 0.0083$ (=0.05/6)

Table 2. Effect of WEIO and EEIO treatment on serum plasma glucose, lipids and lipoprotein levels after 48 hours of treatment.

Variables	Control (n = 10)	Diabetic (n = 10)	WEIO treated (n = 10)	EEIO treated (n = 10)	*p-value
Glucose (mmol/l)	5.65 ± 0.34^{ab}	17.07 ± 1.6^{b}	8.8 ± 2.23^{a}	9.88 ± 1.21	0.000
TC (mmol/l)	3.13 ± 0.75^{cde}	4.74 ± 1.6^{c}	4.31 ± 0.96^{e}	4.20 ± 0.61^{d}	0.017
TG (mmol/l)	1.67 ± 0.63^{fgh}	3.13 ± 1.13^{g}	3.18 ± 0.63^{f}	3.10 ± 0.77^{h}	0.002
HDL (mmol/l)	1.68 ± 0.21	2.00 ± 0.44	2.14 ± 0.44	1.92 ± 0.28	0.123
LDL (mmol/l)	1.25 ± 0.35^{jkl}	1.94 ± 0.46^{j}	1.84 ± 0.22^{l}	1.84 ± 0.14^{k}	0.000

EEIO - ethanol extract of Inonotus Obliquus, WEIO - water extract of Inonotus Obliquus, TC - total cholesterol, TG - triglyceride, LDL - low-density lipoprotein, HDL - high-density lipoprotein. *Kruskal-Wallis test with $p < 0.05$, ^{abcdefghjkl}pairwise comparison using Wilcoxon test with $p < 0.0083$ (=0.05/6)

Histological observations

Histopathological observations (Figure 1) illustrated the pancreatic sections of the normal control group rats stained with H&E showed normal architecture of the pancreatic acini and islet of Langerhans. In addition, the islets of Langerhans were scattered between the acini, they were rounded or oval in configuration, and alpha cells were peripherally located while the ß cells were situated centrally. The sections of the pancreas in the untreated diabetic group showed abnormal shapes, necrotic areas, and vacuolation in islet cells, morphologically confirming the diabetes model.

In diabetic WEIO-treated rats, the pancreatic sections showed partially recovered pancreatic islets, but some degeneration of the β cells in the center were observed. Also, EEIO-treated group morphology demonstrated partial recovery of the pancreas, and the quantity of vacuolated islets cells was reduced.

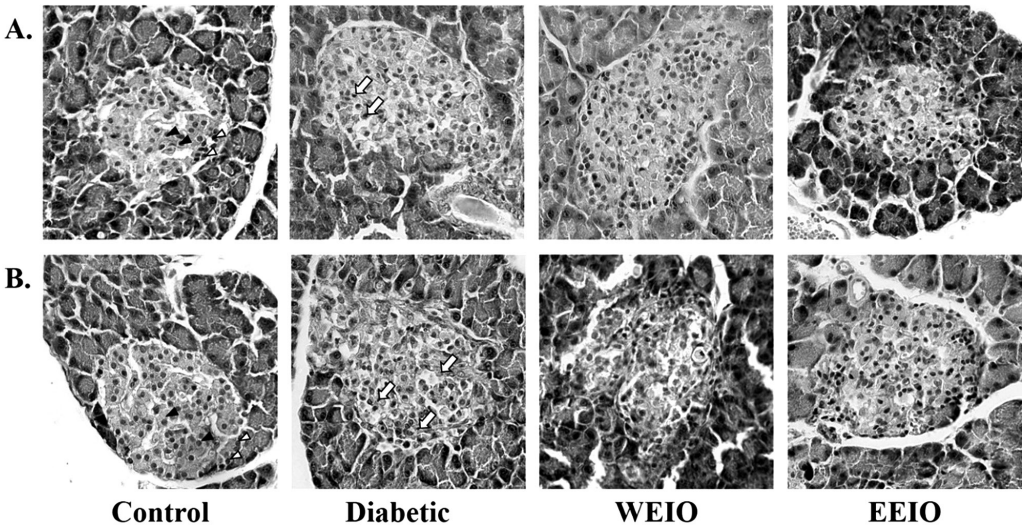

Figure 1. E. Protective effects of native Mongolian IO against alloxan-induced damage to the pancreas. Photomicrograph of the pancreatic section (H and E, × 400) at (A) 24 hours and (B) 48 hours after treatment with ethanol extract of Inonotus Obliquus (EEIO) and water extract of Inonotus Obliquus (WEIO). Open triangle - α-cells, closed triangle - ß-cells, white arrow - necrotic cells.

Discussion

We demonstrated the potential use of native Mongolian IO for diabetes treatment. We showed that extracts of IO could preserve β cell function. IO's treatment effects may result in earlier stages of diabetes and may be an important area for future research in alternative and complementary medicine.

Type 1 diabetes is an autoimmune disease caused by β cell failure of the pancreas. In contrast, hyperinsulinemia occurs in the early stages of type 2 diabetes. Due to hyperinsulinemia in type 2 diabetes, insulin resistance occurs, and this type is characterized by resistance to insulin. Currently, various animal models for diabetes mellitus are used for testing antidiabetic effects, including chemical, pancreatectomy and genetic manipulations. Alloxan monohydrate and streptozotocin are the most commonly used chemicals for inducing animal model diabetes mellitus [27]. Alloxan is less expensive and more readily available than streptozotocin [28]. Alloxan is injected by different administration routes and causes injury in rodent pancreas β cells. The rat is also more sensitive to the agent, making it easier to use as an alloxan-induced type 1 diabetic model than other small mammals such as rabbits and mice [28]. It also is the most economical.

IO has potentially anticancer and antioxidant effects [24]. Previous publications have indicated that IO activates the regeneration of β cells, and prevents irreversible changes and reduces the blood glucose levels by an antihyperglycemic effect in streptozotocin-induced type 2 diabetic mice [29]. We have compared the WEIO and EEIO groups after 24 and 48 hours following treatment with IO with diabetes. In our study, both WEIO and EEIO groups of rats had a significant reduction in blood glucose. This result may have occurred due to possible increased insulin secretion from β cells (Figure 1), and IO's potential antihyperglycemic effects should be explored further in more sophisticated experimental studies [29]. Diabetes is also strongly associated with dyslipidemia, and therefore it is crucial to address breaking this associated problem with safe and efficient extracts [30].

We observed blood lipid-lowering effects of IO in our study. Both water or ethanol extracts of Mongolia IO reduced cholesterol, TG, LDL and increased HDL levels. At 24- and 48-hours after treatment with IO, the cholesterol, TG, and LDL levels of EEIO and WEIO rats decreased. Since HDL carries cholesterol and TG from peripheral tissue to the liver [31], we believe that the cholesterol and TG reduction were related to increases of HDL levels.

Current results indicate in 24 and 48 hours after treatment, Mongolian IO 500 mg/kg minimized and improved the changes associated with pancreatic rat alloxan diabetes. Fewer necrotic changes were observed in Langerhans islet cells in the EEIO groups than in WEIO groups. The polysaccharides isolated from IO reportedly play an important role in the injured pancreatic tissue's recovery [21, 29, 32]. Therefore, IO therapy could potentially result in tissue-level regeneration in the pancreas.

In this study, we had several limitations. We experimented with only short-term outcomes investigation using water and ethanol extracts of IO that is commonly practiced. Future experiments should explore longer-term effects in other domains, including insulin tolerance and underlying mechanisms of diabetes. In addition, preclinical investigations studying supplements to treat type 2 diabetes mellitus are rare for rodent models in resource-limited countries, including Mongolia.

Conclusions

Simple ethanol and water extracts of native Mongolian IO have demonstrated antihyperglycemic effects via enhancing the Langerhans islet cell function and preserving normal islet tissue. Furthermore, these extracts reduce the blood glucose level and dyslipidemia. Extracts of native Mongolian IO are readily producible, economical, and an alternative and complementary medicine approach to treat diabetes.

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