Pancreas-protective effects of chlorella in STZ-induced diabetic animal model: insights into the mechanism

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ABSTRACT

The aim of this study is to examine the effect of intragastric administration of chlorella (1 g/kg body weight) for a period of 30 days to treat normal and diabetic male Wistar rats. Diabetes was induced by intraperitoneal injection of streptozotocin (STZ) (60 mg/kg - 1 body weight). A significant (p < 0.05) reduction of blood glucose level in diabetic chlorella-treated rats was observed compared to diabetic untreated. Chlorella increased the number of glutathione-positive cell in diabetic rats compared to untreated diabetics. Chlorella administration increased the percentage of insulin secreting pancreatic beta cells both in normal and diabetic treated rats. Percentage of glucagon producing alpha cells of the pancreas were reduced both in normal and diabetic chlorella-treated rats. Chlorella-induced regenerative ability on pancreas was mediated by up-regulation of Ki67 and down-regulation of P53 and by its potent antioxidant ability. The present results suggest that chlorella may play an important role in improving the overall condition of diabetic patients and delay its complication by restoring the function of pancreatic insulin-secreting cells.

Keywords: Chlorella; Diabetes; Glucose; Insulin; Beta Cells.

1. INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder affecting approximately 4% of the population worldwide and it is expected to increase to 5.4% in 2025 [1]. It is also characterized by hyperglycaemia and associated with an absolute or relative deficiency in the insulin action and/or secretion [2]. The vast majority of cases of diabetes fall into two broad etiopathogenetic categories. In type 1 diabetes, patients suffer an absolute deficiency of insulin secretion. However, in the more prevalent category type 2 diabetes, the cause is normally a combination of resistance-to-insulin action and an inadequate compensatory insulin-secretory response [3,4]. This results in severe metabolic imbalances and physiologic changes in many tissues, where oxidative stress plays an important role in the etiology [5]. The levels of the reactive oxygen species (ROS) as oxidative stress marker in pancreatic islets of diabetic rats was found to be increased [6].

Oxygen free radical activity can initiate peroxidation of lipids, which in turn stimulates glycation of protein and inactivation of enzymes. There is convincing experimental and clinical evidence that the generation of ROS is increased in both types of diabetes. Normally, the level of oxidative stress is modulated by antioxidant defense systems [7]. Diabetes-linked alterations in antioxidant defense systems have been demonstrated, including alteration in the activities of antioxidant enzymes such as catalase (CAT) and impaired the reduced glutathione (GSH) metabolism [8]. Increases in oxidative stress markers in pancreatic islets in experimental diabetic rats have also been reported [3]. Supplementation with natural products rich in free radical scavengers and antioxidants may facilitate the regeneration of β-cells and protect pancreatic islets against the cytotoxic effects of streptozotocin [2].

ROS-mediated DNA damage has attracted much attention because of its possible relationship to pathological processes such as carcinogenesis and aging [9]. A role of ROS has also been demonstrated as a cause of
type 1 diabetes mellitus induced by chemicals such as alloxan and STZ in experimental animals [10]. The diabetogenic agent STZ enters the pancreatic β-cell via a glucose transporter-GLUT2 and causes alkylation of DNA. Furthermore, STZ induces activation of polyadenosine diphosphate ribosylation and nitric oxide release. As a result of STZ action, pancreatic β-cells are destroyed by necrosis [11]. In adult rats, 60 mg/kg is the most common dose of STZ used to induce type 1 diabetes [4].

Despite the ongoing introduction of new hypoglycemic drugs, diabetes (and its related complications) remains a major global medical problem and a more successful treatment is of great interest, but yet to be discovered. Modern drugs, including insulin and other oral hypoglycemic agents such as thiazolidinediones, biguanides and sulphonylureas, control blood glucose level as long as they are regularly administered, but they also produce many undesirable side effects [1]. Recently, diabetic healthcare professionals have shown a substantial interest in considering complementary and alternative approaches to minimize the burden of complications associated with this disease. Toward that end several experimental studies have been conducted to evaluate either raw or purified active ingredients of an array of natural products. Nowadays, clinical treatment of diabetes targets insulin deficiency, resistance and the declination of pancreatic β-cell function [4].

Natural antioxidants have gained a great interest of food scientists, nutrition specialists and even the general public, as they reduce the risk of chronic diseases and promote human health [12]. Unicellular green alga chlorella is an eukaryotic microalga. Several lines of evidence have shown the anti-atherogenic effects of chlorella in animal models [13]. Chlorella has also been reported to possess anti-oxidative, anti-inflammatory and anti-tumor properties both in vitro [14], and in vivo [15,16]. Administration of chlorella has been shown to improve glucose challenge both in normal and STZ-induced diabetic mice [17] and its hypoglycemic effect was shown both in alloxan-induced and STZ-induced diabetic animals (STZ) [18].

Despite the chlorella’s protective effects on insulin secretion, its effect on the integrity of the pancreas of rats is yet to be carefully investigated in drug-induced diabetic models. The present study was undertaken to demonstrate the insulin-conserving potential and preventive nature of chlorella on pancreatic β-cells damage in STZ-induced diabetic rats, by biochemical, histological and immunohistochemical techniques.

2. MATERIALS AND METHODS

2.1. Animals and Chlorella

Male Wistar rats weighing approximately 200 grams (8 weeks old) were used throughout this study. Rats were obtained from the Faculty of Medicine and Health Sciences, United Arab Emirates University breeding colony. The study was approved by the UAEU Animal Research Ethics Committee. All rats were housed at temperature (25°C) and humidity controlled rooms and 12 hours light and dark periods. The animals were fed on a standard rat chow and tap water ad libitum. Chlorella was purchased as tablets from Wakunaga of America Co., LTD. Mission Viejo, CA, USA.

2.2. Induction of Experimental Diabetes

Diabetes was induced in rats by a single intraperitoneal (ip) injection of STZ (Sigma, Poole, UK) at a dose of 60 mg/kg body weight [19]. The STZ was freshly dissolved in citrate buffer (0.5 M, pH 4.5). Five days after STZ injection, the fifteen hours fasting rats were tested for diabetes by using a drop of blood from the tail end of each rat. The blood glucose estimations were made by One Touch Ultra, Glucometer (LifeScan, Johnson & Johnson, CA, USA) for each individual rat. The average fasting blood glucose levels of the selected diabetic rats were equal to 310 mg/dl.

2.3. Experimental Design

Diabetes Following the diagnosis of diabetes, both age matched control and STZ-induced diabetic rats were divided up into four groups each containing ten rats. The rats were orally administered daily with chlorella (1 g/kg body weight) for 30 days. The age matched control rats received the vehicle (water) over the same period of time. The four groups were as follows: 1) Group 1: normal untreated control. 2) Group 2: normal chlorella treated rats. 3) Group 3: diabetic untreated control. 4) Group 4: diabetic chlorella treated rats.

2.4. Plasma Glucose Measurement

The fasting blood glucose level was measured at starting time for each individual rat of all groups then every ten days till the last 30 days. Blood samples for fasting glucose measurement were drawn from the tail vein using One Touch Ultra blood glucose meter, Life Sciences, USA. The mean of the fasting blood glucose for each of the experimental groups was measured during the experiment period.

2.5. Intraperitoneal Glucose Tolerance Test (IGTT)

At the end of the 30 days of the treatment, all rats in normal and diabetic groups were subjected to ip glucose tolerance test, after an overnight fasting (of about 15
hours). Each rat was given ip glucose load, 2 g/kg body weight [20]. The blood glucose measurements were made at 0 (prior to the glucose load), 30, 60, 120 and 180 min after the glucose load [21].

2.6. Tissue Processing

At the end of the experiment, all the rats from each group were humanely killed under general anesthesia by diethyl ether. A mid-line abdominal incision was made and the pancreas was rapidly removed. Representative fragments were taken from different parts of the pancreas and used for histological studies.

2.7. Histology and Immunohistochemistry

Ten rats from each group (normal and diabetic) were used for the experiments. The isolated pancreas was trimmed free of adherent fat and connective tissues, cut into small pieces (2 mm³), fixed overnight in freshly prepared 10% neutral phosphate buffered formalin, sectioned at 5 µm thicknesses and stained with hematoxylin-eosin and avidin-biotin-peroxidase complex staining system. Sections were examined for normal morphological study and for β-positive cells of insulin secretion and α-positive cells of glucagon secretion under Olympus BX 41 (Japan) light microscope.

2.8. Immunofluorescence

Isolated pancreatic tissues were retrieved, fixed and embedded in paraffin. Sections of about 5 µm thickness were de-paraffinized in xylene, hydrated in descending concentration of ethanol (3 min each) and washed 3 times in PBS for (5 min each). After washing in PBS, the tissue was marked around with a Dako pen to prevent solutions draining away from the tissue section. The staining procedure was started by incubating the sections with blocking reagent (bovine serum albumin in phosphate buffered saline) for 30 min. Thereafter, the blocking reagent was drained off and appropriate dilution of primary antibodies were applied and incubated at 4°C for 24 hours. On the following day, sections were incubated at the room temperature for 1 hour. The slides were then washed 3 times in PBS (5 min each), incubated with secondary antibodies conjugated with TRITC (Jackson Laboratory, USA) for 1 hour and washed in PBS (3 times 5 min each). Sections were finally mounted in CITI-Floure mounting media and photographed with Ziess Axiophot Fluorescence Microscope.

2.9. Image Analysis

Antigens under investigation were analyzed using ImageJ software (1.410—by Wayne Rasband, National Institute of Health, USA). Percentage of antigen positive-cells in the islets of Langerhans of pancreatic tissue was determined using an automated image analysis program implemented by the authors using Matlab (version 7.00, 1984-2004, The Math Works Inc.). It gives positive-cells by calculating the percentage ratio of areas immuno-stained areas with insulin-, glucagon- (DAKO, USA) while, CAT- and GSH- (Sigma, USA) or with P53- and Ki67- (Thermo Fisher Scientific, Massachusetts, USA) positive cells to the whole islet area on a slide [22]. The percentages relative to control (which was set as zero) were calculated. Evaluation was performed in four different islet sites for each of the four experimental groups (normal untreated, normal chlorella treated, diabetic untreated and diabetic chlorella treated; n = 10). The mean and standard error (M ± SEM) value was then calculated giving the percentage positive cells for each group.

2.10. Statistical Analysis

All values were expressed as mean ± standard error of the mean (SEM). Student’s t-test was used to analyze the significance of differences between mean values and different groups were assessed using SPSS statistic analysis software. Values with P < 0.05 were accepted as significant comparing control and treated samples.

3. RESULTS

3.1. Effect of Chlorella Treatment on Blood Glucose Levels of Normal and Diabetic Rats

Figure 1 shows the time course of fasting blood glucose (FBG) level of normal and diabetic rats after treatment with chlorella compared to untreated rats. The FBG was almost unchanged in normal chlorella treated rats compared to normal untreated rats. A slight reduction of FBG levels was detected in diabetic treated rats compared to untreated diabetic rats.

3.2. Effect of Intraperitoneal Glucose Tolerance Test on Chlorella Treated Normal and Diabetic Rats

The results for the IGTT of normal and diabetic rats were shown in Figure 2. The results showed that normal rats treated with chlorella had no significant difference in IGTT from normal untreated rats. However, the diabetic chlorella treated rats showed a beneficial effect on glucose tolerance specially a significant decrease (P < 0.05) in blood glucose levels of diabetic treated rats after 60 min compared to diabetic untreated rats following the glucose load.
3.3. Effect on Antioxidant Enzymes

To assess the potential of chlorella as an antioxidant, the activities of CAT, and GSH in pancreas were measured. Figures 3 and 4 depict the levels of activities of antioxidant enzymes (CAT and GSH), in pancreas of experimental rats. Diabetic untreated rats showed marked decrease in activities of antioxidant enzymes, whereas, diabetic chlorella-treated rats showed a clear elevation of the activities of antioxidant enzymes significantly compared to diabetic untreated rats.

Panel E of Figures 3-4 and 6-9 show the results of image analyses for CAT, GSH, insulin, glucagon, Ki67 and P53 immunoreactive cells of pancreatic islets by immunohistochemical intensity as described in Materials and Methods.

Figure 1. Time course effect of chlorella (1 g/Kg body weight) on blood glucose levels of (a) normal and (b) diabetic rats compared to untreated control rats. (Data are mean ± SEM, n = 10).

Figure 2. Time course effect of treatment with chlorella (1 g/Kg body weight) on IGTT of (a) normal and (b) diabetic rats compared to untreated control rats. (*P < 0.05) was a significant difference in diabetic treated rats compared to untreated diabetic rats. (Data are mean ± SEM, n = 10).

Figure 3. Representative images of rat pancreatic tissue sections stained for CAT by immunofluorescence. Micrographs showing the distribution of CAT positive-cells in the pancreatic islet of normal and diabetic rats. (a) CAT-positive cells in normal untreated rat pancreas; (b) CAT-positive cells in normal chlorella treated rat pancreas; (c) CAT-positive cells in diabetic untreated rat pancreas; (d) CAT-positive cells in diabetic treated rat pancreas. Pictures were taken at 400× magnification. (e) The effect of treatment with chlorella on percentage of CAT-positive cells in pancreatic islets of normal and diabetic rats. The data show a significant difference (**) P < 0.01) in diabetic treated rats compared to untreated diabetic rats. (Data are mean ± SEM, n = 10).
3.4. Effect of Chlorella Treatment on Percentage of Insulin Positive Cells of Normal and Diabetic Rats

Figure 5 shows a normal histopathological architecture of the pancreatic islets and the ordinary distribution of pancreatic acini in normal rats. On the other hand, the diabetic control shows disruption of the acini with small atrophic islet cells. Treatment of diabetic rats with chlorella leads to expansion and enlargement of islet cells.

Figure 6 shows the effect of chlorella on insulin-positive cells in normal and diabetic rats. The results show that treatments of normal and diabetic rats with chlorella increase the percentage of insulin secreting positive β-cells in both normal and diabetic treated rats.

However, normal treated rats showed marked significant increase (P < 0.01) of insulin secreting positive cells when compared to normal untreated rats. The results also showed a significant increase (P < 0.05) of insulin secreting cells in diabetic treated rats compared to diabetic untreated rats. The histograms also confirmed the abundance of insulin-positive cells in pancreatic islet of Langerhan’s in treated rats than in untreated ones in both normal and diabetic groups.

3.5. Effect of Chlorella Treatment on Percentage of Glucagon Positive Cells of Normal and Diabetic Rats

Effects of chlorella on immunoreactive positive α-glucagon cells in normal and diabetic rats were shown in Figure 7. The results showed that treatments of normal and diabetic rats with chlorella decrease the percentage of glucagon secreting positive α-cells in all normal and diabetic treated rats. A significant (P < 0.001) decrease in α-cells was detected in diabetic treated rats compared to diabetic untreated rats. Less prominent α-cells in pancreatic islet of Langerhan’s in treated normal and diabetic rats than in untreated ones was also quantified (Figure 7(e)).

3.6. Effect of Chlorella Treatment on Proliferation and Apoptosis in Pancreatic Islets

In the present study, the expression level of a replication marker Ki67 was chosen as an established replication-associated protein with a broad clinical application (Figure 8). The diabetic-associated significant reduction (P < 0.01) of Ki67 was alleviated with chlorella treatment.

On the other hand, P53 was chosen as an apoptotic marker in the pancreatic islets. P53 expression was sig-
4. DISCUSSION

Diabetes mellitus is a severe endocrine disease that includes a group of disorders of varying etiology and pathogenesis. The management of diabetes is an escalating global predicament and a cure has yet to be discovered. Drugs such as insulin and other hypoglycemic agents control blood glucose level only when they are regularly administered. These treatments, however, have several disadvantages [23]. Fortunately natural products, among other alternative medicines, offer a similar degree of efficacy sans many problematic side effects. STZ is selectively toxic to the β-cells in the pancreatic islets and it eventually induces diabetes in adult rats [10]. STZ-induced hyperglycemia has been described as a good experimental model to study diabetes mellitus. Its administration to rats showed an increase in the blood glucose levels and a decrease in the plasma insulin levels [2].

Hyperglycemia, the most prevalent characteristic of diabetes mellitus, is in itself very harmful for diabetic patients. The elevated blood glucose levels are thought to lead to cell death through oxidative stress induction that occurs as a common sequel of diabetes-induced modification of sugar moieties on proteins and lipids [3,24]. Hyperglycemia increases oxidative stress through the overproduction of ROS, which results in an imbalance between free radicals and the antioxidant defense systems of the cells. The administration of chlorella decreases blood glucose concentration in diabetic rats demonstrating the blood glucose-controlling ability of chlorella and its role as an essential trigger for the liver to revert to its normal anti-oxidative ability.

Glucose uptake by muscle is increased after chlorella administration. This may be the explanation for positive glucose tolerance test in normal mice reported in previ-
Figure 8. Immunohistochemical evaluation of Ki67 expression in pancreas. (a) Control rat showing normal structure and Ki67-positive β cells; (b) Rats administered with chlorella alone showing normal structure and Ki67-positive β cells; (c) streptozotocin-diabetic rats showing the decrease in Ki67 immunoreactivity and the number of immunoreactive β cells; (d) Diabetic rats treated with chlorella showing increase of Ki67 immunoreactivity and the number of immunoreactive β cells. Pictures were taken at 400× magnification. (e) The effect of treatment with chlorella on percentage of Ki67-positive cells in pancreatic islets of normal and diabetic rats. The data show a significant increase (**P < 0.01) in diabetic treated rats compared to untreated diabetic rats. (Data are mean ± SEM, n = 10).

Figure 9. Immunohistochemical evaluation of P53 expression in pancreas. (a) Control rat showing normal structure and P53-positive β cells; (b) Rats administered with chlorella alone showing normal structure and P53-positive β cells; (c) streptozotocin-diabetic rats showing the increase in P53 immunoreactivity and the number of immunoreactive β cells; (d) Diabetic rats treated with chlorella showing decrease of P53 immunoreactivity and the number of immunoreactive β cells. Pictures were taken at 400× magnification. (e) The effect of treatment with chlorella on percentage of P53-positive cells in pancreatic islets of normal and diabetic rats. The data show a significant decrease (**P < 0.01) in diabetic treated rats compared to untreated diabetic rats. (Data are mean ± SEM, n = 10).

ous study [17]. Glucose uptakes showed no difference in soleus muscles between normal and STZ mice, however, the uptakes were enhanced after chlorella administration. Therefore, increasing the chlorella-induced glucose uptake in the soleus muscles may explain its ameliorating effect on hyperglycemia [18]. At 60 min after intraperitoneal glucose challenge, blood glucose of diabetic rats treated with chlorella was significantly lower compared to untreated diabetic rats, however, blood glucose level was not significantly different at other time points. The inability of the pancreas to significantly lower glucose levels at other time points could be due to the fact that the newly formed pancreatic beta cells may not be mature enough to release effective insulin capable of lowering blood glucose.

Antioxidant protective systems against ROS and the breakdown products of peroxidized lipids and oxidized protein and DNA are provided by many enzyme systems such as CAT and GSH. Oxidative stress in diabetes is coupled with a decrease in the antioxidant status, which can increase the deleterious effects of free radicals [25]. CAT is one of the two major scavenging enzymes that remove radicals in vivo. A decrease in its activity can lead to an excess availability of free radicals like H₂O₂ which in turn generate OH⁻ resulting in initiation and propagation of lipid peroxidation [3]. In this study, activity of CAT was restored in chlorella treated diabetic rats compared to diabetic untreated group. In addition, GSH was significantly increased in diabetic chlorella treated rats compared to diabetic untreated suggesting that chlorella has free radical scavenging activity. Chlorella may then enhance the activity of the antioxidant protective system which may exert a beneficial effect against pathological alterations caused by reactive oxygen species and thus preserving pancreatic β-cell integrity.

Changes in blood glucose and in numbers of insulin-secreting cells reflect abnormalities in β-cells function and structure. STZ impairs glucose oxidation and decreases insulin biosynthesis and secretion. It also generates ROS, which contribute to DNA fragmentation and evoke other deleterious changes in β-cells [1].
The present histopathological investigation showed that diabetic pancreas revealed necrotic and fatty infiltration of the islet cells. Chlorella treated diabetic pancreas showed near normal structure of pancreatic islet cells. Also here, chlorella treatment preserved pancreatic β-cell integrity. The current immunohistochemical examination showed that pancreatic β-cells are destroyed by STZ whereas chlorella partially prevented degeneration of β-cells. Chlorella treatment increased the area of insulin immunoreactive β-cells significantly. In the STZ-diabetic rats, a significant decrease in insulin immunoreactivity was observed. However, after the treatment of chlorella, the insulin immunoreactivity was improved and an increase in the number of immunoreactive β-cells was observed in comparison to the diabetic group. According to the immunohistochemical results obtained, chlorella may have the ability to enhance insulin sensitivity and regenerate the β-cells of diabetic rats. Anti-hyperglycemic effect of natural product extracts is generally dependent upon the degree of β-cell destruction [26]. The present findings suggest that chlorella may regenerate β-cells and has protective effect on β-cells from glucose toxicity since the morphological changes seen in β-cells of diabetic rats are compatible with those observed when islets are exposed to high concentrations of glucose. The anti-hyperglycemic effect of chlorella may be mediated through insulin secretion from the remnant β-cells and increase insulin sensitivity [27]. Aucubin, another natural product isolated from Plantago asiatica, was shown to stimulate glucose intake into cells and thus lowering the blood glucose level [3]. A number of other natural products have also been observed to exert hypoglycemic activity through insulin release stimulatory effects [1]. Similarly, administration of chlorella to diabetic rats markedly reduced STZ-induced number of glucagon-positive cells in the pancreas.

In the present study, the expression level of Ki67 was chosen as an established replication-associated protein with a broad clinical application, e.g. in the histological grading of malignant tumours. Previous studies reported tight correlations between Ki67 and many other replication markers in a variety of tumours and suggested that Ki67 is suitable for the quantitative determination of β-cell replication in pancreatic tissue sections [28].

Even though the overall expression Ki67 was significantly up regulated following chlorella treatment in diabetic rats, the expression of Ki67 protein was restricted within individual cells of the pancreatic islets. This suggests that the expression of other proliferation markers may indeed be associated with the process of β-cell replication. In this context, MCM7 protein has been described to peak at the G1/S-transition point in various cell types [29], while PCNA expression reaches its maximum during S-phase [30]. Ki67 is expressed at very low levels in late G1 and early S [31] but increases during cell cycle progression, reaching its maximum in late S and G2 [32]. By these means, determination of the expression of more than one replication marker may not only allow for the discrimination between replicating cells and quiescent cells, but may also yield additional information about its current stage of cell cycle progression [28].

In this study, we also demonstrate that chlorella attenuates apoptosis and increases cell survival in pancreatic β-cells. This effect was mediated by down regulating p53. Pancreatic β-cell failure plays a key role in the pathogenesis of type 2 diabetes. Although the exact mechanism underlying β-cell destruction is not known, it has been suggested that both hyperglycemia and hyperlipidemia contribute to β-cell destruction. Both glucotoxicity and lipotoxicity are important in the pathogenesis of type 2 diabetes because they lead to interference with insulin signal transduction and thus insulin resistance on the one hand and β-cell destruction on the other [33]. The functional consequences of glucotoxicity and glucolipotoxicity for β-cells include induction of cell death by apoptosis [34].

The present study suggests that the antihyperglycemic and antioxidant effects of chlorella preserve endocrine pancreas and protect pancreas from STZ-induced toxicity. Increased insulin secreting cells after treatment with chlorella positively altered the deranged carbohydrate metabolism in the STZ-induced diabetic rats. The protective effects of chlorella in experimental diabetes are mediated by decreasing oxidative stress, enhancing proliferation and cell survival and resulting in the preservation of pancreatic β-cell integrity.

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