Cytological and Histochemical Studies in Rat Liver and Pancreas during Progression of Streptozotocin Induced Diabetes and Possible Protection of Certain Natural Antioxidants

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Abstract

Background: Diabetes mellitus is a major endocrine disorder and growing health problem in most countries. Diabetes manifested by experimental animal models exhibit high oxidative stress due to persistent and chronic hyperglycemia which increases the generation of free radicals. Streptozotocin (STZ) provides an animal model of type 1 diabetes. Thereby depleting the activities of antioxidative defense systems with alteration of antioxidant activities of enzymes such as green tea and curcumin.

Aim: Biochemical histological and histochemical investigations were carried on to reveal the effect of STZ on the liver and pancreas cells. Natural antioxidants were used as a new way for ameliorated diabetic effect on the cells.

Material and methods: Diabetes was induced by a single intraperitoneal injection of freshly prepared STZ dissolved in 0.05 M of sodium citrate buffer, pH=4.6, (STZ; 45 mg/kg B.wt.). Three days after degeneration of beta cells, diabetes was induced in all animals. After induction of diabetes, diabetic and normal animals were kept in metabolic cages separately. Green tea (EGCG) and curcumin are used as a natural antioxidant to improve the disorders and structural changes induced by STZ. Cellular and histochemical investigations were carried on the changes induced in pancreatic and hepatic tissues. Body weight, levels of serum glucose and insulin were calculated, and compared. For a microscopic study of degeneration of both hepatocytes and pancreatic cells of diabetic rats, tissue samples from diabetic and treated rats were collected, and pathologically examined.

Results: Our investigations revealed that there was a detectable amelioration on the injuries induced by STZ on both hepatocytes and pancreatic cells using green tea or curcumin with a detectable dose level. Also it can be observed that the ameliorated effect induced was a time dependant. Conformation of these results from histochemical detection of glycogen and DNA contents were detected by PAS and feulgen reactions.

Conclusion: Curcumin and green tea looks to have a powerful effect against diabetic cell injury induced in both rat liver and pancreas. The ameliorating effect seems to be time dependant.

Keywords: Biochemistry; Liver; Pancreas; Pathology; STZ; Histochemistry

Introduction

Diabetes is a chronic disease that is relatively common throughout the world. In recent decades, various epidemiological studies have been carried out on prevalence of diabetes mellitus all over the world according to which the population of diabetics was obviously increased according to World Health Organization reports, more than 150 million people throughout the world suffered from diabetes while the mankind has been unable to solve this problem [1].

Administration of 60 mg/kg streptozotocin dose can initiate an autoimmune process that results in the destruction of the Langerhans islets beta cells, ultimately contributing to the toxicity of beta cells. According to this model, oxidative stress is produced under diabetic conditions which possibly cause various forms of tissue damage in patients with diabetes. Diabetes mellitus is a complex and a multifarious group of disorders that disturbs the metabolism of carbohydrates, fats and proteins. It results from shortage or lack of insulin secretion or reduced sensitivity of the tissue to insulin. Several drugs such as biguanides and sulfonylureas are currently available to reduce hyperglycemia in diabetes mellitus [2,3]. Treatment of diabetes starts with a healthy diet and natural antioxidant. The diet should be low in refined sugars and fats. Diet should be high in fibers, grains and legumes (beans, peas). Nuts are good, as onions and garlic for maintaining blood glucose levels coming to this fact. Antioxidants can exert beneficial effects on both liver and pancreatic b-cell function in diabetes. Thus a sufficient supply of antioxidants may prevent or delay b-cell dysfunction in diabetes by providing protection against glucose toxicity. Green tea has protective effect in assisting diabetics, and in particular type 1 diabetic, with the breakdown of blood glucose. Although many plants offer certain medical benefits to humans, many of these claims are unproven scientifically. Research on animals has suggested that, green tea may in fact help to prevent the development of type 1 diabetes, also helping to regulate blood sugar levels in those who already have type 1 diabetes. Type 1 diabetics do not produce...
insulin which breaks down glucose in the blood, but the use of green tea may help in regulating these blood sugar levels and so be beneficial for diabetics [4-6]. Many question related to antioxidant effect of green tea extract remain unanswered. Much more work is of clearly orally administration of green tea extract (1.5%, w/v) produced significantly by reduction of blood glucose level in healthy normal rats. At the same time it had been found that it had a significant hypoglycemic effect in diabetic rats [7,8].

At the same level curcumin found to be the main biologically active photochemical compound of turmeric. It is extracted, concentrated, standardized and researched. Curcumin, which gives the yellow color to turmeric, was first isolated almost two centuries ago, and its structure as diferuloylmethane was determined in 1910. Curcumin (diferuloylmethane) is a naturally occurring yellow pigment isolated from the rhizomes of the plant Curcuma longa (Linn) found in south Asia and is a potent antioxidant agent and free radical scavenger. Extensive research within the last half a century has proven that, its renowned range of medicinal properties, once associated with turmeric, are due to curcumin, a polyphenolic compound derived from dietary spice turmeric possesses diverse pharmacologic effects including anti-inflammatory, antioxidant, anti proliferative and anti-angiogenic activities [9-12]. Along with being an inhibitor of lipid peroxidation it is also an inhibitor of Nitric Oxide Syntheses (NOS) over expression and of nuclear factor kappa B activation. Curcumin treatment at 12 week can exert beneficial effect in diabetes mellitus, regarding the improvement of pancreatic islets. The islets of Langerhans neogenesis is characterized by the presentation of small islets increase in numbers nearly the ducts and no insulin [10]. The efficacy of curcumin has been widely observed in reducing various diabetic secondary complications such as diabetic nephropathy/renal lesions retinopathy wound healing and reduction of advanced glycation end products.

Further studies evaluate the effect of curcumin that could influence pancreatic islets recuperation after the damage induced by STZ. It had been found that in the diabetic mice which were induced by STZ, alkylating agent, DNA was damaged in beta-cell and subsequent inhibition of insulin biosynthesis and secretion [11,12].

Considering the antihyperglycemic properties and antioxidant activities of green tea extract, and curcumin [13-17], this study was designed to evaluate comparative ameliorating effects of them both in hepatic and pancreatic injury induced by STZ.

Material and Methods
Animals
The present study was done on healthy adult albino male rats in the weight range of (150-200 gm), selected from an inbred group housed in specially designed cages and maintained under standard conditions of temperature (23 ± 1°C) and humidity of (55-60%) with a 12-hour light and 12-hour dark cycle for at least one week before use. Rats were grouped to (4) groups each of (10) rats on the basis of initial weight and kept in individual cages; all animals consumed standard rodent diet and tap water ad libitum. Clinical monitoring of the animals was also performed to evaluate body weight and blood glucose and insulin levels weekly. All animals were cared for according to the guiding principle in the care and use of animals.

Natural Antioxidants
Green tea extracts (EGCG): Green tea extract was obtained in the form of tablets. Each tablet contain 300 mg of green tea dry extract. In the present study green tea extract was prepared by dissolving the tablets in distilled water at dose level 45 mg/1 ml/rat/day. It was administered daily by gavages.

Curcumin: Curcumin product was prepared as a suspension solution at dose level 300 mg/ml H2O. Each experimental animal gavage 1 cm/day by steel tube at dose level 300 mg/ Kg/rat.

Chemicals
Streptozotocin (STZ) was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Induction of diabetes
Diabetes was induced by a single intraperitoneal injection of freshly prepared Streptozotocin (STZ) (Sigma chemical company), stored at 4°C temperature and protected from environmental extremes (STZ; 45 mg/kg B.wt.) dissolved in freshly prepared 0.05 M of sodium citrate buffer, pH=4.6 [18]. Fasting rats must measure their normal blood glucose level before STZ injection (zero-time), then measured again after 48 hr of STZ injection to be sure that the rats became diabetic, their weight also must be determined before and after injection.

STZ-injected groups considered to be diabetic when blood glucose values were above (250 mg/dl). The blood glucose level was determined by glucose oxidase method using a one touch basic plus glucometer (Lifescane Ltd., California, USA).

Experimental Design
Animal treatments
Treatments were initiated soon after establishment of diabetes 3 days after administration of Streptozotocin.

Animal groups and Experimental design
Sixty four healthy adult male albino rats weighing from 200-220 gm were used in this study, all animal were housed under standard conditions and received human care in compliance with institutional guidelines, and they were kept under good ventilation, fed on standard laboratory diet, and supplied with water ad libitum. They were divided into four groups, each consisted of 16 rats:

- **Group I**: considered the normal control; feeding on normal diet (injected i.p. with citrate buffer).
- **Group II**: considered the model control (diabetic group): group of animals suffer from diabetes.
- **Group III**: (green tea diabetic groups): groups of animals suffer from diabetes supplemented with green tea (EGCG) and examined after 3 and 6weeks.
- **Group IV**: (curcumin supplemented diabetic groups): groups of diabetic animals supplemented with curcumin and examined after 3 and 6 weeks.

Biochemical preparation
**Blood glucose level detection:** Blood glucose level and weight of every rat determined every week. Blood sample collected from ocular orbit vein by heparinized hematocrit capillary tubes. The blood glucose level was determined by glucose oxidase method using a one touch basic plus glucometer (Lifescane Ltd., California, USA).

**Insulin estimation:** Insulin was assayed in the Medical Service Unit of the National Center for Radiation Research and Technology.
Center (by ELISA kits) according to the method of Byersdorfer et al. [19] based on the sandwich principle.

Histopathological preparation

The sacrificed animals were quickly dissected. Sample of the liver and pancreas were removed and fixed in 10% neutral formalin for 24 hours followed by washing, dehydration in ascending grades of alcohol, clearing in xylene and embedding in hard paraffin. Sections were cut by the microtome at (5μ thick), stuck on clean slides and allow drying. Sections were deparafinized in xylene and hydrated to water through descending series of ethyl alcohol. Staining was performed using hematoxylin and counterstained by 0.5 aqueous eosin for nucleus and cytoplasm examination and investigated by light microscope [20,21]. All stained sections were dehydrated through ascending series of ethanol (100%, 90%, 70% respectively) purified in xylene and finally mounted with DPX.

Histochemical preparation

Deoxyribonucleic acid (DNA): DNA was histochemically determined by applying Feulgen's technique [21]. This method depends on the treatment of fixed tissues by mild hydrolysis with (N-HCl) at 60°C which could release the aldehyde group from the deoxypentose sugar of DNA. Following hydrolysis, the tissues were washed and then transferred to Schiff’s reagent which reacts with the exposed aldehyde groups to produce a reddish purple dye in the nuclear chromatin alone.

General carbohydrates PAS reaction: General carbohydrates were demonstrated following the application of Periodic Acid Schiff’s (PAS) technique [22]. This method involves the oxidation of carbohydrate material with 0.5% periodic acid which leads to the liberation of aldehyde that can be histochemically localized by combination with Schiff’s reagent to give a substituted dye which is magenta in color.

Results

Table 1, showed remarkable differences in body weight, glucose and insulin levels among the four groups. (Chart 1) showed decrease in body weight in group 2 which is diabetic, a gradual significant increase in the body weight can be recognized in both treated groups.

Blood glucose of STZ diabetic rat significantly increased when compared to normal control, while blood glucose of treated diabetic rat groups III, IV were significantly decreased when compared with diabetic group and nearly normal when compared to normal control (Chart 2). Meanwhile Chart 3 reviled statistical decrease in serum insulin level
of group 2 when compared with normal control. On the other hand groups 3 [green treated diabetic group] showed slight increase in the insulin level on the third week and becoming more significant on the sixth week. At the same level, curcumin shows ameliorating effect on the third week and comes to be nearly normal on the sixth week.

Pathological Observations

Plate (I) liver: Examined sections of normal control rat group I showed that most of the cells contain a central rounded nucleus while some binucleated. The blood sinuses are present between the cords. The sinusoidal endothelium is formed of endothelial lining cells and the phagocytic kupffer cells (Figure 1 G1). Liver sections from diabetic rats group II showed severe injury illustrated in mononuclear cell infiltrate extending through hepatic tissue. Kupffer cell appeared engulfing debris and hyperplasia of bile duct (Figure 2a G2). Obviously fatty change [lipoma] which is also a common feature could be seen in (Figure 2b G2). Examined sections of group III which is treated with green tea showed graduate restoration of hepatocytes and blood sinuses. Most of the cell were healthy and seem to be normal (Figures 3 G3 and 4 G3). Moreover pancreatic cells of group IV that is treated with curcumin declared that curcumin have much better effect after 3 weeks the cells showed healthy structure.

Plate (II) pancreas: Pancreatic section from group I showed normal pancreatic structure [normal islets of Langerhans and normal acini tissues] illustrated in Figure 1 G1. Pancreatic sections from group II which is treated with STZ showed disorganization of the structure of the endocrine and exocrine cells illustrated in less of Langerhans cells with damaged and necrotic pancreatic acini (Figure 2 G2). On the other hand sections from group III that is treated with green tea at two interval times 3 and 6 weeks, there was a gradual restoration of pancreatic endocrine cells, degeneration of some of the pancreatic acini was still observed, (Figures 3 G3 and 4 G3). More over pancreatic cells of group IV that is treated with curcumin declared that curcumin have much better effect after 3 weeks the cells showed healthy structure.

Plate 1: Photomicrograph of liver sections of albino rats (H&E stain) from the following groups: Figure [1G1]: (X 400). Figure [2 G2] (X 400). Figure [3 G 3 ] (X 400). Figure [4 G3] (X 400). Figure [5 G4 ] (X 400). Figure [6 G4] (X 400). (CV) central vein. (S) dilated blood sinusoid.

Plate 2: Photomicrograph of pancreas sections of albino rats (H&E stain) from the following groups: Figure [1 G1]: (X 400). Figure [2 G2 ] (X 400). Figure [3 G3] (X 400). Figure [4 G3] (X 400). Figure [5 G4] (X 400). Figure [6 G4](X 400).

Plate 3: Photomicrograph of liver sections of albino rats (PAS stain) from the following groups: Figure [a G1](X 400). Figure [b G2] (X 400). Figure [c G3] (X 400). Figure [d G3] (X 400). Figure [e G4] (X 400). Figure [f G4] (X 400).
Meanwhile the most interesting aspect of the examined pancreatic sections treated with curcumin after 6 weeks showed enlarged atrophied islet, infiltration of inflammatory cells and blood cells through Langerhans islets Figure 6 G4.

Histochemical observations

Charbohydrate detection [glycogen] [PAS]

Plate (III) liver: Glycogen contents of rat liver decreased in diabetic animals when compared to normal control animals but these levels increased to near normal after treatment with green tea and curcumin. Examined liver section of group I showed normal pattern distribution of glycogen granules (Figure a G1). Liver section of group II showed marked depletion in the glycogen granules (Figure b G2). Meanwhile this depletion became increased in green tea treated animals of group III, this seem to be time dependant (Figures c G3 and figure d G3). The results obtained from curcumin treated animals in group IV declared that, curcumin treatment in the first 3 weeks were in normal pattern while it became increased in content in the next 3 weeks (Figures e G4 and f G4).

Plate (IV) pancreas: On the other hand examined diabetic green tea treated animals showed gradual elevation in DNA content granules (Figures c G3 and figure d G3). While sections from group IV, curcumin treated animals 3 weeks showed irregularity in the size of the nuclei most of the nuclei were faint and polymorphic with normal appearance of chromatin content (Figures e G4 and figure f G4).

DNA content

Plate (V) liver: Liver sections of group I showed normal distribution of DNA granules most of the nuclei were in the same sizes (Figure a G1), while DNA of liver sections from diabetic group II, showed marked decrease in DNA content (Figure b G2). Liver sections from group III,
pancreatic sections that, treated with green tea there was a gradual increase in DNA content 3 and 6 weeks after treatment compared to normal while sections that were treated with curcumin were retain its normal pattern at the 6 week of treatment (Figures g G1, h G2, I G3, j G3, k G4, l G4).

Discussion

Diabetes mellitus is a complicated group of disorders characterized by hyperglycemia that increase the global prevalence in the present century. Diabetes mellitus type 1 (type 1 diabetes) is an autoimmune disorder caused by lymphocytic infiltration and betacells destruction within the pancreatic islets of Langerhans. The pancreatic beta-cells are lost in numbers and volume, then severe permanent insulin deficiency results. Oxidative stress is produced under diabetic conditions and possibly causes various forms of tissue damage in patients with diabetes. Examine the involvement of oxidative stress in the progression of pancreatic B-cell dysfunction in type 1 diabetes, it had been observed that, by most estimates, it is now the most common cause of liver disease. However, evidence suggests that, oxidative stress and free radicals play an important role in the pathogenesis of diabetes mellitus and diabetic complications [23]. The STZ diabetic mice exhibited persistent hyperglycemia which is the main diabetogenic factor and contributes to the increase in oxygen free radicals by autoxidation of glucose. Hyperglycemia also generates reactive oxygen species, which in turn, cause lipid peroxidation and membrane damage. Diabetes increases oxidative stress in many organs, especially in the liver [24].

Liver is one of the most important organs that maintains blood glucose levels within normal limits thus enhancement of blood sugar yield to imbalance of oxidation-reduction reactions in hepatocytes, so that, hyperglycemia through increasing in AGES (advanced glycation end products) facilities free radicals production via disturbance in ROS (reactive oxygen species) production . Hence, it reveals that, diabetic hepatic injuries results from several agents and is not controllable only via inhibition of hyperglycemia. Namely, although in early stages of diabetes, tissues injuries are induced via hyperglycemia but its progress in latter stages is not related to hyperglycemia. Therefore, monitoring of blood glucose levels solely is not sufficient in retarding diabetes complications. Thus, a suitable drug must have both antioxidant and blood glucose decreasing properties [25-30]. In accordance to our results blood glucose level of treated diabetic rat were significantly decreased when compared with diabetic group and nearly normal when compared to normal control. On the other hand serum insulin level showed statistically decrease when compared with normal control, diabetic groups showed none significant change [31,32]. These finding confirmed the opinion that Curcumin and green tea in diabetes rats cause, degradation of liver glycogen and increase gluconeogenesis, while glucose utilization is inhibited. Glucose 6-phosphatase increases in the liver facilitating glucose release into the blood. Green tea is considered to be anti-inflammatory, antioxidative, antiinmutagenic, and anticarcinogenic [33-36]. Other interesting observation found was the differences in body weight among diabetic and treated groups, diabetic group showed decrease in body weight. This may be due to lack of insulin in the blood, the sugar cannot enter inside the cell, thus increasing the percentage of sugar in the blood. The body tries to get rid of excess sugar by excretion in the urine. With many secretion of urine will lead to the reduced amount of water in the body and this reduced weight. Many studies reviles that, this loss of weight may be related to significant hypoglycemic effect in diabetic rats, glucose intolerance could arise from either a defect in insulin secretion as in case of insulin dependent diabetes (Type I) or a defect in insulin resistance (receptor or post-receptor defect) as in case of non insulin dependent diabetes mellitus (Type II) [37-39]. On the other hand a gradual significant increase in the body weight was observed in the other treated groups (groups III, IV). This may be due to the retained levels of glucose and insulin levels in green tea and curcumin treated animals [36]. In many previous morphological studies there were many pathological changes, degenerated hepatocytes with polymorphic nuclei, dilated sinusoids and mononuclear cell infiltrate extending through hepatic tissue. Kupfer cells appeared engulging debris and hyperplasia of bile duct is also found as pathological finding in diabetic animal [27]. This confirms our finding observed in this work.

The interesting aspect is the fatty changes in centrilobular portions of the livers in diabetic group [40]. With green tea treatment in diabetic rats no considerable fatty change were observed indicating the protective effect of green tea against hepatic complications of diabetes [41]. However, other pathologic findings were that Green tea and curcumin found to have graduate restoration and ameliorating effect and it is time dependant. It is clearly observed that, curcumin give more ameliorating effect. It has been reported the effectiveness of curcumin in reducing secondary complications in STZ induced diabetic animals [11]. Moreover, the curcumin has been demonstrated in prevention isolated betacell death and dysfunction induced by STZ [10,11].

On the other hand histopathological examination from this work, pancreatic diabetic rats revealed reduction in number of islets, degeneration of B cells, hydropic degeneration, clumping of b cells, pyknosis and necrosis showed the change in the shape of cells, this can be attributed to the partial damage of streptozotocin to some beta cells. Lower dose of streptozotocin produced an incomplete destruction of pancreatic beta cells even though rats became permanently diabetic [28]. Also other previous studies found that, pathological examination of STZ diabetic rats showed reduced number of islets of Langerhans degranulation of B cells, hydropic degeneration, pyknosis and necrosis, these findings are in accordance with our findings which revealed less of Langerhans cells, presence of damaged pancreatic acini pyknosis and necrosis. Our studies also come to the fact that excess use of curcumin in diabetic animal may cause reverse effect, this can easily seen in examined section of group IV treated for 6 weeks [31].

Some studies have reported that streptozotocin enters the beta cells via a glucose transporter (GLUT 2) and causes alkylolation of DNA damage induces activation of poly ADP-ribosylation, a process that is more important for the diabetogeneity of streptozotocin than DNA damage itself. Poly ADP-ribosylation leads to depletion of cellular NAD+ and ATP. Enhanced ATP dephosphorylation after streptozotocin treatment supplies a substrate for xanthine oxidase resulting in the formation of super oxide radicals. Consequently, hydrogen peroxide and hydroxyl radicals are generated. Furthermore streptozotocin liberates toxic amounts of nitric oxide that inhibits aconitate activity and participates in DNA damage. As a result of the streptozotocin action, beta cell undergoes destruction by necrosis. Other studies carried on diabetic liver indicated that cytotoxic effects of streptozotocin are dependent upon DNA alkylatation by site-specific action with DNA bases and by free-radical generation during streptozotocin metabolism [42-44]. The finding of this study agrees with these findings which showed depletion in DNA in both liver and pancreatic cells. In conclusion, our study provides clear evidence of pancreatic islets growth to respond to curcumin and green tea treatment in diabetic mice at 3, 6 weeks. More over our observation is appropriate for the further molecular studies and search of new factors to evaluate the implicated mechanisms in the
pancreatic islets, modifications in the future that could lead to provide a new therapeutic of diabetes mellitus.

References