Anti-diabetic activity of oryzanol and its relationship with the antioxidant property
Somuvara B. Ghata & Shital S. Panchal

Abstract Experimental evidence suggests that oxidative stress induced by streptozotocin (STZ) damage the pancreatic beta cells and produce hyperglycemia in rats. The current study was initiated to investigate the effects of oryzanol (OZ), a commercially-important bioactive phytochemical isolated from crude rice bran oil (cRBO) against experimental diabetes as well as the antioxidant potential of the drug. OZ was isolated by a two-step solvent crystallization process from cRBO, which was extracted from fresh rice bran by hexane mediated soxhlet extraction. Oral administration of OZ (50 and 100 mg/kg) reduced the blood glucose level in normal and in STZ (45 mg/kg, intravenous) diabetic rats in both single and multidose study. Oxidative stress produced by STZ was found to be significantly reduced by OZ when compared to control rats, as evident from a significant decrease in the extent of lipid peroxidation (LPO) and increased levels of enzymatic anti-oxidants such as superoxide dismutase (SOD) and reduced glutathione (GSH) in the liver. The findings indicate that OZ possesses the potential to effectively ameliorate the oxidative stress induced by STZ and produce a reduction in blood glucose levels. However, further experiments at the clinical level are warranted to confirm the utility of OZ in the therapeutic management of diabetes mellitus.

Keywords Oryzanol · Antioxidants · Normal rats · Diabetes · Hyperglycemia

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Introduction

Free radicals, generated as by-products of normal cellular metabolism, have been implicated in the etiology of several diseases such as liver cirrhosis, atherosclerosis, cancer and diabetes. The compounds that can scavenge free radicals have tremendous potential in ameliorating these disease conditions [1]. Antioxidant defense mechanisms, involving both enzymatic and non-enzymatic strategies, thus play an important role in the protection of the human body against damage by reactive oxygen species (ROS) [2]. Increased oxidative stress has been suggested as mechanism underlying diabetes and its related complications [3]. In diabetes, increased production of free radicals, especially ROS, caused by persistent hyperglycemia, can initiate peroxidation of lipids, which in turn stimulates non-enzymatic glycation of protein, inactivation of enzymes and alterations in the structure and function of collagen, basement and other membranes which collectively produces the late diabetic complications [4]. Oxidative stress in diabetes coexists with an altered cellular redox status and a sharp reduction in the antioxidant defense status, which can subsequently increase the deleterious effects of free radicals [5].

For several decades, the β-cell-specific toxin streptozotocin (STZ), an analogue of GlcNAc, has been used to establish animal models of diabetes, by virtue of its ability to induce oxygen free radicals and selectively destroy the pancreatic insulin secreting β-cells, leaving less active cells resulting in a diabetic state [6, 7]. Supplementation with exogenously administered non-toxic antioxidants may exhibit a chemoprotective role in diabetes [8]. Antioxidants such as
vitamins C and E have demonstrated reduction in oxidative stress in experimental diabetes [9]. Moreover, supplementation with vitamin C has also been shown to lower glycosylated hemoglobin levels in diabetic patients [10].

Considering the major impact of the increasing global prevalence of diabetes due to the absence of effective and affordable interventions, a wide range of oral medicines are currently being used. However, various adverse effects such as liver problems, lactic acidosis, diarrhea and high rates of secondary failures have been associated with the available anti-diabetic medications [11]. Therefore, the search for more affordable agents from natural origin that retain the therapeutic efficacy and are devoid of side effects has now become the major focus of scientists and researchers.

In recent times, there has been an emerging interest in the scientific community to evaluate both crude and isolated natural products in experimental studies and utilize the phytochemicals for their potential therapeutic usage in minimizing the risk of various diseases [12, 13]. In the light of this perspective, rice bran oil (RBO) has been the focus of attention because of its balanced fatty acid profile and its rich content of bioactive anti-oxidative polyphenols such as, ferulic acid, its esterified derivatives such as oryzanols (OZ), tocopherols, tocotrienols and other associated phenolic compounds [14]. The major components of the saponifiable fraction of RBO include oleic and linoleic acids, while the principal component of the unsaponifiable fraction is OZ [15]. The antioxidant components, more than its fatty acids, contribute to the beneficial effects of RBO [16].

OZ, an important constituent of crude rice bran oil (cRBO), is a mixture of ferulic acid (4-hydroxy-3-methoxycinnamic acid) esters with phytosterols [17] and primarily extracted from rice bran. A number of therapeutically useful biological activities such as reduction of cholesterol levels, modulation of the pituitary secretion, inhibition of the gastric acid secretion, antioxidant action and inhibition of the platelet aggregation have been reported for OZ [18].

Furthermore, previous studies investigating the supplementary effects of OZ, as a constituent of experimental diets, on the blood glucose level in diabetic mice, reported a lower concentration of fasting blood glucose and blood glucose area from glucose tolerance test as compared to the control group [19]. Subsequent studies by Ohara et al [20] indicated that hydroxycinnamic acid derivatives such as OZ might regulate adiponectin secretion by the inhibition of NF-kB activation. Since adiponectin is positively correlated with insulin sensitivity, it was proposed that OZ might be effective in ameliorating type 2 diabetes. A recent study by Son et al [21] illustrated that OZ could reduce the risk of high-fat diet-induced hyperglycemia via regulation of insulin secretion and hepatic glucose-regulating enzyme activities. However, available literature shows that no systematic and scientific investigation has been carried out to verify the claims on the anti-diabetic activity of OZ against diabetogenic agents like STZ and its relationship with its anti-oxidant properties.

The objective of this investigation was to ascertain the scientific basis for the use of OZ in the treatment of diabetes mellitus. Therefore, the present study was designed to investigate the protective effect of OZ, isolated from cRBO, on lowering the blood glucose levels and various markers of oxidative stress (i.e. lipid peroxidation, glutathione and superoxide dismutase in liver) in STZ-induced diabetic rats.

Materials and methods

Drugs and chemicals

Rice bran was procured from Suryodaya Rice Mills, Ahmedabad, Gujarat, India through their milling process. Streptozotocin was purchased from Sigma chemicals (St. Louis, Mo, USA). Standard OZ was purchased from Tokyo Chemical Industry Co., Ltd. Tokyo, Japan. All other chemicals used were of analytical reagent grade and were obtained from commercial sources.

Extraction of RBO

Fresh rice bran obtained from the local rice mill was stored in a refrigerator before use. Crude RBO was extracted from rice bran (50 g) by soxhlet extraction for 3 h using hexane as the solvent. The extracted crude oil was stored at −5 °C and subsequently analyzed for the various physicochemical parameters, such as organoleptic characters, specific gravity, viscosity, moisture content, saponification value, unsaponifiable matter, wax content, iodine value, acetyl value, acid value, hydroxyl value, ester value and peroxide value using various standard official methods, the results of which have been published previously [22].

Isolation of OZ from cRBO

The isolation of OZ from cRBO was achieved by a two-step crystallization process previously described by Zullaikah et al [23] with some modifications. In the first step of isolation, the OZ-rich product was concentrated in the liquid phase by solvent crystallization using methanol/acetone (7:3, v/v). In the second step, the OZ-rich product was kept at ambient temperature for 24 h. After that, hexane was added as an anti-solvent and kept at 5 ± 1 °C for 48 h. Under optimal operational conditions, supplemented by considerable savings of both time and solvents, white OZ crystals with a purity of 94.07 % and recovery of 55.61 % were obtained. The isolated OZ was identified with respect to the standard by melting
point determination, thin-layer chromatography (TLC), UV-visible spectrophotometry and high-performance liquid chromatography (HPLC), the results of which have been published recently [24].

Experimental animals

Adult wistar rats of either sex weighing 250–300 g were procured from the central animal facility of the Institute of Pharmacy, Nirma University, Ahmedabad. The animals were maintained at controlled temperature as well as humidity and fed with standard diet and water provided ad libitum. The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC) of Institute of Pharmacy, Nirma University, as per the guidance of committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

Dose calculation

Considering the typical daily dosage of OZ as 500 mg/day in humans, oral test doses for rats were selected as 50 and 100 mg/kg, based on the dose translation formula on the basis of the body surface area [25, 26].

Effect of OZ on blood glucose levels in normal rats (single and multi dose study)

Adult wistar rats of either sex were divided into three groups of six rats per group. Group-I animals were administered with 4 % Tween-80 solution through oral intubation, which served as normal control (NC). Group II and III were given OZ orally at dose levels of 50 mg/kg (OZ-50) and 100 mg/kg (OZ-100), respectively in 4 % Tween-80 solution.

In single dose study, after administration of single doses of OZ (OZ-50 and OZ-100), blood samples were collected from the retro-orbital plexus just prior to and 1, 2, 4 and 6 h intervals. Serum was separated and glucose levels were estimated by glucose oxidase-peroxidase (GOD/PDO) enzymatic method of Trinder [27].

For multidose study, the same groups of normal animals were continued with the same dose levels once daily, up to 11 days. The glucose levels of all the animals were measured on 3, 5, 7, 9 and 11th day, respectively.

Effect of OZ on serum glucose levels in STZ-induced diabetic rats (single and multi dose study)

Diabetes was induced in adult wistar rats of either sex by a single intravenous injection of 45 mg/kg of STZ dissolved in citrate buffer (0.1 M, pH -4.5) [28]. Serum glucose levels were checked after 72 h. Animals with serum glucose levels >300 mg/100 ml were considered diabetic and were used for the study [29]. The diabetic rats were divided into three groups of six rats per group. Control diabetic rats (Group-I) were given 4 % Tween-80 solution. Groups II and III were given OZ orally at dose levels of 50 mg/kg (OZ-50) and 100 mg/kg (OZ-100), respectively in 4 % Tween-80 solution.

In single dose study, after administration of a single dose, blood samples were collected from the retro-orbital plexus just prior to and 1, 2, 4 and 6 h intervals. Serum was separated and glucose levels were estimated.

For multidose study, the same groups of diabetic animals were continued with the same dose levels once daily, up to 11 days. The blood glucose levels of all the animals were measured on 3, 5, 7, 9 and 11th day, respectively.

Estimation of biochemical parameters

Isolation of liver

On the 11th day, all the animals were euthanasiously sacrificed by cervical dislocation. Liver was collected and was blotted free of blood and tissue fluids. Then it was weighed on balance and the relative weight was calculated.

Preparation of the tissue homogenate for enzyme assay

Liver, kept in cold conditions (pre-cooled in inverted petridish on ice) was removed. It was cross chopped with surgical scalpel into fine slices and was chilled in the cold 0.25 M sucrose, quickly blotted on a filter paper. The tissue was minced and homogenized in 10 mM Tris-HCl buffer, pH 7.4 (10% w/v) with 25 strokes of teflon pestle of glass homogenizer at a speed of 2,500 rpm. The clear supernatant was used for biochemical assays.

Parameters assessed in the liver homogenate

The levels of lipid peroxidation (LPO) products as thiobarbituric acid reactive substances were estimated by the method of Ohkawa et al [30]. Glutathione was estimated by the method of Moron et al [31] based on the reaction of GSH with dithiobis trinitro benzoic acid (DTNB). SOD was estimated by the modified method of Misra and Fridovich [32]. The protein content was determined by Lowry’s method [33].

Statistical analysis

All the values are expressed as mean±S.E.M. Statistics was applied using Graph Pad Prism version 5.0 for Windows, Graph Pad software, San Diego, California, USA. One way ANOVA followed by Tukey’s multiple comparison test was used to determine the statistical significance between
Table 1 Effect of oryzanol (OZ) on serum glucose level in fasted normal rats (single dose)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Serum glucose level (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fasting</td>
</tr>
<tr>
<td>I</td>
<td>Normal Control</td>
<td>78.1±4.8</td>
</tr>
<tr>
<td>II</td>
<td>OZ 50 mg/kg</td>
<td>84.6±4.0</td>
</tr>
<tr>
<td>III</td>
<td>OZ 100 mg/kg</td>
<td>82.8±6.3</td>
</tr>
</tbody>
</table>

Values are mean±SEM, N=6, *represents significance at P<0.05 for glucose level as compared to normal control (Tukey’s multiple comparison test)

various groups. Differences were considered to be statistically significant when P<0.05.

Results

Effect of OZ on serum glucose level

Oral administration of OZ at 50 mg/kg and 100 mg/kg was found to reduce serum glucose level in normal rats both in single and multi-dose study. In the single dose study, the maximum reduction in serum glucose level (49.97 %) was noted at second hour by OZ at 100 mg/kg followed by a 47.76 % reduction by OZ at 50 mg/kg (Table 1). In the multi-dose study from 3rd to 11th day, OZ at 100 mg/kg significantly (P<0.05) reduced the serum glucose level, while OZ at 50 mg/kg significantly (P<0.05) reduced the serum glucose level from 7th to 9th day (Table 2).

The effect of OZ at 50 mg/kg and 100 mg/kg in STZ induced diabetic rats is shown in Tables 3 and 4. The fasting blood glucose levels in STZ induced diabetic rats were 340–400 mg/100 ml. During the single dose study, the results were found to be similar to that of normal animals. Maximum effect was seen within 2–4 h with OZ at 100 mg/kg. A tendency for the serum glucose to increase after 6 h was typically observed. There was a fall in glucose level (13.09 % and 12.38 % respectively) in these rats 4 h after administration of OZ at 100 mg/kg and 50 mg/kg respectively, in the single dose study, although the reductions were not statistically significant.

Similar results were observed upon continuous administration of both the doses of OZ. The serum glucose levels of STZ alone treated group significantly increased from 345.9 to 464.94 mg/100 ml on day 7 after the STZ injection. On the 5th day, OZ at 100 mg/kg showed a significant (P<0.05) reduction in the serum glucose levels, while from the 7th to 9th day, serum glucose levels were significantly (P<0.05) decreased in both the OZ treated groups when compared with the STZ treated control group. Again, on the 11th day, OZ at 100 mg/kg demonstrated a highly significant (P<0.05) serum glucose lowering effect as compared to the STZ treated control group (Table 4).

Effect of OZ on oxidative stress markers

Serum LPO level was elevated significantly in diabetic animals compared to normal animals. Administration of OZ at 50 mg/kg and 100 mg/kg reduced the serum LPO level significantly (P<0.05) on the 11th day (Fig. 1a). The GSH level was found to be low in the STZ treated group, while in both the OZ treated groups, the levels increased significantly (P<0.05) on the 11th day when compared with the untreated diabetic animals (Fig. 1b). The SOD activity was found to be reduced in the liver of animals treated with STZ. SOD values in rats treated with STZ along with OZ at 50 mg/kg and 100 mg/kg were significantly higher (P<0.05) on the 11th day (Fig. 1c).

Table 2 Effect of continued administration of oryzanol (OZ) on serum glucose level in normal rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Serum glucose level (mg/100 ml)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3rd day</td>
</tr>
<tr>
<td>I</td>
<td>Normal Control</td>
<td>77.7±5.4</td>
</tr>
<tr>
<td>II</td>
<td>OZ 50 mg/kg</td>
<td>64.0±6.3</td>
</tr>
<tr>
<td>III</td>
<td>OZ 100 mg/kg</td>
<td>57.4±4.3*</td>
</tr>
</tbody>
</table>

Values are mean±SEM, N=6, *represents significance at P<0.05 for glucose level as compared to normal control (Tukey’s multiple comparison test)
Table 3 Effect of oryzanol (OZ) on serum glucose level in fasted diabetic rats (single dose)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Serum glucose level (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fasting</td>
</tr>
<tr>
<td>I</td>
<td>Diabetic Control</td>
<td>345.9±32.5</td>
</tr>
<tr>
<td>II</td>
<td>OZ 50 mg/kg</td>
<td>400.2±38.2</td>
</tr>
<tr>
<td>III</td>
<td>OZ 100 mg/kg</td>
<td>384.6±46.3</td>
</tr>
</tbody>
</table>

Values are mean±SEM, N0.6, *represents significance at P<0.05 for glucose level as compared to diabetic control (Tukey’s multiple comparison test)

Discussion

The present study indicates that OZ at 50 mg/kg and 100 mg/kg significantly decreased serum glucose levels in normoglycemic and hyperglycemic animals, with a more pronounced effect being observed with the higher dose. In normal rats, administration of OZ at 100 mg/kg showed 36.71 %, 49.97 %, 44.08 % and 31.03 % decline in the blood glucose levels on 1, 2, 4, and 6 h, respectively and 30.67 %, 28.74 %, 30.91 %, 31.88 % and 33.93 % decline in the blood glucose level on 3, 5, 7, 9 and 11 day, respectively. STZ-induced diabetic rats, on the other hand, administered with OZ at 100 mg/kg showed 4.68 %, 10.03 %, 13.09 % and 12.40 % decline in the blood glucose level on 1, 2, 4, and 6 h, respectively and 20.43 %, 28.34 %, 30.62 %, 33.30 %, and 46.07 % decline in the blood glucose level on 3, 5, 7, 9 and 11 day, respectively.

The observed anti-hyperglycemic action of OZ is probably attributed to the marked enhancement of glucokinase (GK) activity and inhibition of glucose-6-phosphatase (G6pase) and phosphoenolpyruvate carboxykinase (PEPCK) in the liver. An increase in the expression of hepatic GK can stimulate the residual pancreatic mechanism, probably by accelerating the utilization of blood glucose for energy production or glycogen storage in the liver, thereby resulting in reduced blood glucose level. A decrease in G6pase and PEPCK activities, on the other hand, indicates a decrease in hepatic glucose production, since they are the key enzymes involved in the regulation of gluconeogenesis and glucose output from the liver [21].

Since oxidative stress is considered to be a key factor in the development of diabetes, anti-oxidants are recognized as its effective treatment options. Naturally occurring phenolic compounds, such as ferulic acid, by virtue of their strong antioxidant activity, has been shown to reduce blood glucose levels in STZ-induced diabetic animals [34]. The diabetogenic properties of STZ, mediated by pancreatic beta cell destruction, induces oxidative stress due to depletion of anti-oxidant scavenger system, thereby resulting in an elevated level of LPO that is a clear manifestation of the promotion of de novo free radicals generation leading to tissue damage [35]. The significant decline in the concentration of LPO in the liver tissue of OZ treated diabetic animals indicates that OZ effectively enhanced the antioxidant potential in vivo, by acting as a strong superoxide radical and singlet oxygen quenchers. Such a finding is in concordance to that reported by other investigators [21].

GSH, a major non-protein thiol in living organisms, is essential to maintain the structural and functional integrity of the cells. In addition to its potent free radical scavenging properties within the islet of β-cell and abilities to conjugate with several electrophilic intermediates that are capable of initiating lipid peroxidation, GSH is an important factor against the progressive destruction of the β-cell following partial pancreatectomy and acts as the physiological co-substrate of the conjugating enzyme system [36, 37]. Depletion of GSH results in enhanced LPO leading to increased GSH consumption and can be correlated with the increase in the level of oxidised glutathione (GSSG). In the current study, the diabetic animals registered lowered levels of GSH reflecting its increased utilization owing to

Table 4 Effect of continued administration of oryzanol (OZ) on serum glucose level in fasted diabetic rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Serum glucose level (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3rd day</td>
</tr>
<tr>
<td>I</td>
<td>Diabetic Control</td>
<td>415.9±41.6</td>
</tr>
<tr>
<td>II</td>
<td>OZ 50 mg/kg</td>
<td>408.1±28.1</td>
</tr>
<tr>
<td>III</td>
<td>OZ 100 mg/kg</td>
<td>306.0±39.2</td>
</tr>
</tbody>
</table>

Values are mean±SEM, N0.6, *represents significance at P<0.05 for glucose level as compared to diabetic control (Tukey’s multiple comparison test)
Fig. 1  a Effect of oryzanol (OZ) on lipid peroxidation (LPO) level in liver tissues of control, diabetic and treated rats. * The values are significant compared to the following (Tukey’s multiple comparison test): normal control (*P<0.05), # The values are significant compared to the following: diabetic control (P=0.05), NC normal control, DC diabetic control, OZ-50 oryzanol 50 mg/kg treatment, OZ-100 oryzanol 100 mg/kg treatment. b Effect of oryzanol (OZ) on reduced glutathione (GSH) level in liver tissues of control, diabetic and treated rats. * The values are significant compared to the following (Tukey’s multiple comparison test): normal control (*P<0.05), # The values are significant compared to the following: diabetic control (P=0.05), $ The values are significant compared to the following: oryzanol 50 mg/kg treatment (P<0.05), NC normal control, DC diabetic control, OZ-50 oryzanol 50 mg/kg treatment, OZ-100 oryzanol 100 mg/kg treatment. c Effect of oryzanol (OZ) on superoxide dismutase (SOD) level in liver tissues of control, diabetic and treated rats. * The values are significant compared to the following (Tukey’s multiple comparison test): normal control (*P<0.05), # The values are significant compared to the following: diabetic control (P<0.05), NC normal control, DC diabetic control, OZ-50 oryzanol 50 mg/kg treatment, OZ-100 oryzanol 100 mg/kg treatment.
oxidative stress while, restoration of normal levels of GSH in OZ treated diabetic rats coincided with a significant decline in lipid peroxidation. This observation is in agreement with the findings proposed by the earlier investigators [21].

The antioxidant enzyme SOD, along with glutathione peroxidase (GPX) and catalase (CAT) constitute a mutually supportive team of defense against ROS and plays an important role in alleviating cellular stress. SOD is a metalloprotein and is the first enzyme involved in the antioxidant defense by virtue of scavenging the superoxide radical through its conversion to hydrogen peroxide and molecular oxygen [38]. In the present study, decline in the activities of antioxidant enzymes such as SOD in diabetic rats indicate the extent of free radical mediated damage due to hyperglycemia. It is now evident that an imbalance between free radical production and antioxidant defenses leads to the generation of oxidative stress resulting in deregulation of cellular functions, which can be alleviated by a potent antioxidant drug [39]. The maintenance of SOD levels in OZ administered rats unravels the efficacy of the drug in nullifying the oxidative insult elicited by STZ induced diabetes. Similar observations have been reported in previously published literature [40].

The current study suggests that diabetic animals are exposed to oxidative stress and OZ like natural antioxidants can partially reduce the imbalances between the generation of ROS and the scavenging enzyme activity, thereby strengthening the endogenous antioxidant defenses and restoring the optimal balance by neutralizing the reactive species. In accordance with these results, OZ could be used as a supplemental anti-oxidant therapy and may be beneficial for correcting hyperglycemia and thereby alleviating diabetic complications due to LPO and free radicals. However, longer duration studies on chronic models are warranted in order to confirm the current findings.

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Conflicts of interest None

References

Young inflorescence of Cocos nucifera contributes to improvement of glucose homeostasis and antioxidant status in diabetic rats

R. S. Renjith ☉ T. Rajamohan


Abstract Enhanced oxidative stress and changes in oxidant capacity observed in clinical and experimental diabetes, are thought to be the aetiology of chronic diabetic complications. The aim of the study was to investigate whether Cocos nucifera inflorescence improves glucose homeostasis and antioxidant status, 20% w/w of inflorescence containing diet were given to diabetic Sprague Dawley rats for 45 days. Diabetes was induced in rats by alloxan administration (150 mg/kg bodyweight) intraperitoneally. Treatment with inflorescence significantly lowered blood glucose levels thereby preventing steep onset of hyperglycemia which was observed after alloxan administration. It maintained glucose tolerance and glycosylated hemoglobin close to the values observed in normal control rats. In addition, the levels of serum total cholesterol and triglycerides were also lowered. Antioxidant enzymes registered a decline in their activity in diabetic rats, thus revealing the damaging effects of free radicals generated due to alloxan administration. But their activities were reverted towards near-normal range in inflorescence-supplemented animals. Histopathological analysis of pancreas of diabetic rats showed altered morphology, which was restored to near-normal in inflorescence-treated rats. Oxidative damage in various tissues of diabetic rats as evidenced by marked elevation in the levels of thiobarbituric acid reactive substances was nullified by inflorescence treatment, indicating its antioxidant efficacy in resisting oxidative damage.

Keywords Alloxan · Antioxidant · Cocos nucifera inflorescence · Diabetes mellitus · Homeostasis · Oxidative stress

Introduction

Free radicals have been implicated in the pathogenesis of diabetes mellitus (DM). Hyperglycemia in DM may directly or indirectly contribute to excess formation of free radicals, which ultimately leads to oxidative stress [1]. Enhanced oxidative stress and changes in antioxidant capacity, observed in both clinical and experimental diabetes mellitus are thought to be the aetiology of chronic diabetic complications [2]. Implication of oxidative stress in the pathogenesis of diabetes is suggested not only by oxygen free-radical generation, but also due to nonenzymatic protein glycosylation, auto-oxidation of glucose [3], alteration in antioxidant enzymes [4] and lipid peroxide formation.

Alloxan is a cytotoxic glucose analogue, which selectively destroys pancreatic islets β-cells. Alloxan-induced DNA fragmentation in pancreatic islets and cell damage has been attributed to the production of toxic free radicals [5]. Thus, alloxan model was considered suitable for the study of experimental diabetes, in which free radicals might have a central role. Despite the introduction of hypoglycemic agents from natural and synthetic sources, diabetes and its secondary complications continue to be a major problem in the world. Current drugs used for the treatment of DM involve different mechanisms for bringing down blood glucose levels in normal or glycemic conditions but are associated with several side effects; hence there is need for effective, safe and better oral hypoglycemic agents [6]. Many indigenous Indian medicinal plants have been found to be useful to successfully manage diabetes [7].

Cocos nucifera (L.), belongs to the family Arecaceae, known as coconut palm, is attributed to possess various medicinal properties and culinary applications. Studies carried out using the coconut kernel protein and fiber are found to possess antidiabetic and hypoglycemic properties in experimental animals [8, 9]. In Cocos nucifera, the flowers
and flower bearing ramifications are together called inflorescence. In young inflorescence, the spikelets lie very close to the main axis (peduncle) and the whole is tightly packed. The watery sap that drips from immature inflorescence is used as a natural drink in tropical countries. In Ayurveda, Cocos nucifera inflorescence (CnI) is often used to cure backache. But the biochemical effect of CnI in controlling the complications of diabetes-induced oxidative stress has not been worked out. The present study is an attempt in this direction. In this study, the effect of dietary supplementation of inflorescence on diabetes and resultant oxidative stress has been investigated in alloxan induced diabetic rats.

Materials and methods

Collection and preparation of CnI

Young inflorescence of coconut palm (West Coast Tall variety) harvested from Kerala University campus was used for the study. The material was identified at Department of Botany in the University and a voucher specimen (No. KUBH 5795) was deposited in the herbarium. CnI was then pulverized using an electric laboratory blender. It was then dried at 55 °C in a hot air oven and the dry weight was taken and used for in vivo experiment.

Chemicals

Alloxan was purchased from Sigma Chemicals, St. Louis, USA. Kits for glucose, total cholesterol and triglycerides estimation were purchased from Agappe Diagnostics, Thane, India. All other chemicals used were of highest analytical grade possible.

Animals and experimental design

All the animal cares and procedures were according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

The experimental design and protocol used in this study was approved by Institutional Animal Ethics Committee (IAEC). One month old male Sprague Dawley rats (150–200 g BW) bred in our department animal house were used for the study. Diabetes was induced in 20 rats by injecting them with alloxan (150 mg/kg BW) dissolved in normal saline intraperitoneally after fasting the animals for 24 h. The rats were then kept on 5% glucose solution for the next 24 h to prevent drug induced hypoglycemic phase. All the survived rats (65%) were found to be diabetic after 72 h. Rats with a blood glucose level ≥250 mg/dL were considered to be diabetic.

All the animals were randomly divided into four groups (N0 6). Group I rats served as the normal control, while group II rats which were non-diabetic were given 20% CnI (CnI control group). Group III rats served as the diabetic control. Diabetic rats in group IV were fed with 20% CnI (treated group). In an earlier dose response study conducted in our laboratory, we used three doses (10, 20 and 40% w/w of CnI) to compare and maximum effect was obtained from 20% and that dose was selected for the study (unpublished result) (Table 1). Group I and group III animals received groundnut oil and glucocomman along with laboratory rat chow (VRK Nutritional Solutions, Pune, India.) (Table 2). Group II and IV animals received a diet which includes CnI, corn starch and casein along with laboratory rat chow. The different dietary components were given to each group so as to adjust the dietary intakes (% w/w) of all the four groups to be the same as shown in Table 3. The animals were housed individually in polypropylene cages in a room maintained at 25±10 °C with a 12 h light and 12 h dark cycle.

The experiment lasted for a period of 45 days. After the experimental period, animals were fasted overnight and sacrificed by intraperitoneal injection of thiopentone sodium (>40 mg/kg BW). Serum was separated from the blood samples collected through cardiac puncture and tissues were collected immediately and homogenized for various estimations.

Serum glucose and lipid levels

Estimation of serum glucose was done using a commercial kit based on glucose oxidase/peroxidase enzymatic method

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal control</th>
<th>Diabetic control</th>
<th>Diabetic treated with 10% CnI for 45 days</th>
<th>Diabetic treated with 20% CnI for 45 days</th>
<th>Diabetic treated with 40% CnI for 45 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Glucose</td>
<td>86.76±8.3</td>
<td>242.28±23.2*</td>
<td>151.12±14.4</td>
<td>123.8±11.8**</td>
<td>129.67±12.4**</td>
</tr>
<tr>
<td>(mg/dl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>4.87±0.36</td>
<td>8.08±0.77*</td>
<td>6.53±0.62**</td>
<td>6.04±0.57**</td>
<td>6.54±0.77**</td>
</tr>
<tr>
<td>Total Hb (gm %)</td>
<td>14.6±1.3</td>
<td>11.8±1.0*</td>
<td>12.9±1.1</td>
<td>13.4±1.2**</td>
<td>13.2±1.2**</td>
</tr>
</tbody>
</table>

Values are mean±SD of six rats. *P<0.05 compared to normal control; **P<0.05 compared to diabetic control.

Serum glucose is expressed in mg/dL; HbA1c is expressed as g% of total Hb; Total Hb is expressed in g/dL.
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<tr>
<td>Total carbohydrates</td>
<td>11.85*</td>
<td>56.98</td>
</tr>
<tr>
<td>Crude protein</td>
<td>15.25*</td>
<td>22.04</td>
</tr>
<tr>
<td>Crude fat</td>
<td>6.50*</td>
<td>2.43</td>
</tr>
<tr>
<td>Crude fiber</td>
<td>59.56*</td>
<td>11.84</td>
</tr>
<tr>
<td>Total ash</td>
<td>6.84*</td>
<td>6.71</td>
</tr>
</tbody>
</table>

* Values are mean of three estimations

[10]. Serum cholesterol and triglycerides were estimated by cholester oxidase method [11] and GPO-PAP-ESPAS method [12] respectively.

Serum glycosylated hemoglobin (HbA1c) and oral glucose tolerance test (OGTT)

Glycosylated hemoglobin was measured by the method of Eross et al. [13] and expressed as percentage of total hemoglobin. OGTT was performed after 45 days of treatment with Cnl as described earlier [14].

Activities of antioxidant enzymes

Superoxide dismutase and catalase were determined by the method of Kakkar et al. [15] and Maehly and Chance [16] respectively.

Levels of thiobarbituric acid reactive substances (TBARS)

Lipid peroxides were estimated using TBARS by the method of Okhawa et al. [17].

Histopathology of pancreas

For histopathological studies, the pancreas were removed and preserved in 10% formaldehyde. Five micrometer thickness sections were prepared and stained with hematoxylin and eosin (H/E) [18]. Stained sections were qualitatively evaluated using a photo microscope (Zeiss Axioscope 2 plus, USA) equipped with a Canon Zoom Browser EX digital camera (Japan).

Statistical analysis

All the data were statistically evaluated with Statistical Package for Social Sciences (SPSS) version 11.5. (SPSS Inc., Chicago, USA). Hypothesis testing methods included one way analysis of variance (ANOVA) followed by Duncan’s post hoc multiple variance test. ‘P’ values of less than 0.05 were considered to indicate statistical significance. All the results were expressed as Mean±SD for six animals in each group.

Results

Effect of Cnl on serum glucose, HbA1c and OGTT

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Serum lipid levels

Administration of alloxan significantly elevated the lipid profile in the serum of diabetic control compared to the normal animals (Table 4). Administration of Cnl to normal rats had no effect on the total cholesterol, but an improvement in the levels of triglycerides, whereas diabetic rats fed with Cnl showed a significant reduction in the total cholesterol and triglyceride levels.

Antioxidant enzymes and TBARS

There were decreased activities of SOD and CAT in diabetic rats compared to the normal rats (Table 4). Cnl alone fed rats showed almost similar antioxidant capacity as normal animals. The activities of these enzymes in Cnl fed diabetic rats were found to be restored to the normal levels. The levels of TBARS were significantly increased in diabetic rats.
Table 4 Effect of 45 days treatment with Cocos nucifera inflorescence (CnI) on biochemical parameters in alloxan-induced diabetic rats

<table>
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<tr>
<th>Parameter</th>
<th>Normal control</th>
<th>Normal treated with CnI for 45 days</th>
<th>Diabetic control</th>
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</tr>
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<tbody>
<tr>
<td>Serum glucose</td>
<td>74.7±6.7</td>
<td>72.23±6.1</td>
<td>295.27±21.3</td>
<td>156.64±15.3</td>
</tr>
<tr>
<td>HbA1c</td>
<td>4.75±0.37</td>
<td>4.35±0.35</td>
<td>7.55±0.55</td>
<td>5.65±0.40</td>
</tr>
<tr>
<td>Total Hb</td>
<td>14.5±1.3</td>
<td>14.8±1.3</td>
<td>12.4±1.1</td>
<td>13.8±1.2</td>
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<td>Serum cholesterol</td>
<td>68.97±6.32</td>
<td>68.35±6.2</td>
<td>95.74±8.73</td>
<td>77.71±7.0</td>
</tr>
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<td>Triglycerides</td>
<td>89.03±8.1</td>
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<td>Hepatic SOD</td>
<td>13.08±1.2</td>
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<td>Renal SOD</td>
<td>15.64±1.4</td>
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<td>5.93±0.3</td>
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<tr>
<td>Hepatic CAT</td>
<td>75.5±6.7</td>
<td>80.4±7.6</td>
<td>31.3±2.8*</td>
<td>39.0±2.5**</td>
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<tr>
<td>Hepatic TBARS</td>
<td>0.73±0.05</td>
<td>0.72±0.01</td>
<td>2.49±0.22*</td>
<td>1.75±0.14**</td>
</tr>
<tr>
<td>Renal TBARS</td>
<td>2.85±0.24</td>
<td>2.41±0.20</td>
<td>4.72±0.43*</td>
<td>4.30±0.37</td>
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Values are mean±SD of six rats. *P<0.05 compared to normal control; **P<0.05 compared to diabetic control
Serum glucose, cholesterol and triglycerides are expressed in mg/dL; HbA1c is expressed as g% of total Hb; Total Hb is expressed in g/dL; SOD is expressed in Units/mg protein; CAT is expressed in ×10⁻³ is expressed in mM/100 g wet tissue

Compared to normal and CnI alone fed rats (Table 4). CnI feeding decreased the concentration of these lipid peroxidation products to near basal level in diabetic rats, showing its antioxidative effect.

Histopathology of pancreas

Histopathological findings of pancreas of normal control rats revealed normal acini and islets with normal round or elongated structural intactness with their nucleus (Fig. 2a). Normal treated rats also showed the same morphology as control (Fig. 2b). Pancreas of diabetic rats exhibited extensive damage and hypoplasia of Islets of Langerhans and acini in untreated rats was rated as 4+ (Fig. 2c), whereas diabetic rats treated with CnI reduced the dimensions of hypoplasia and restored the cellular population size of islets to almost normal rated as 2+ (Fig. 2d).

Discussion

The present study evaluates the biochemical effect of CnI in alloxan induced diabetes in experimental rats. The results demonstrated that CnI significantly ameliorated the adverse influence of alloxan. Preliminary studies conducted by us revealed the non-toxic nature of CnI. To the best of our knowledge; this is the first report utilizing CnI as antidiabetic and antioxidative phytonutrient. Dietary supplementation of CnI after alloxan treatment resulted in lower serum glucose levels, improved glucose tolerance and lipid profile as compared with rats administered alloxan alone. These changes were accompanied by a significant
The aldehydeic product of lipid peroxidation, malondialdehyde (MDA) is more cytotoxic and stable than reactive oxygen species and reacts quickly with cellular constituents [25]. This in turn may contribute to the disruption of intracellular and membrane redox state of liver and pancreatic β-cells, hence disturbing glucose homeostasis. Dietary supplementation of CnI to diabetic rats reversed all these alterations to the basal levels. Moreover, the results of histopathological examination of the pancreas in experimental rats confirmed that CnI can inhibit β-cell shrinkage and reverse the reduction in the number of β-cells in diabetic rats, thereby maintaining the tissue integrity both in endocrine and exocrine pancreas.

Preliminary investigations revealed that CnI contains proteins, secondary metabolites like polyphenols, flavonoids and also soluble and insoluble fibers (unpublished result). It is suggested that the active principles from plant sources might act by several mechanisms such as stimulating insulin secretion, increasing repair or proliferation of β-cells, enhancing insulin sensitivity and increasing the antioxidative capability [26, 27]. CnI showed antidiabetic effects in experimental rats, which was evident from the biochemical and histopathological findings and that may be mediated via its antioxidative capability and β-cell repair. However, further studies are required to elucidate the detailed mechanism of action of CnI in this regard to develop it as a promising natural antidiabetic drug.
Acknowledgement The financial assistance from KSCSTE, Trivandrum in the form of KSCSTE Fellowship is gratefully acknowledged.

Conflict of interest The authors declared no potential conflicts of interest.

References

1. El-Shenawy NS, Abdel-Nabi IM. Hypoglycemic effect of cleome droserifolia ethanolic leaf extract in experimental diabetes, and on non-enzymatic antioxidant, glycogen, thyroid hormone and insulin levels. Diabetol Croat. 2006;35:15.
Young inflorescence of Cocos nucifera contributes to improvement of glucose homeostasis and antioxidant status in diabetic rats

R. S. Renjith & T. Rajamohan


Abstract Enhanced oxidative stress and changes in oxidant capacity observed in clinical and experimental diabetes, are thought to be the aetiology of chronic diabetic complications. The aim of the study was to investigate whether Cocos nucifera inflorescence improves glucose homeostasis and antioxidant status, 20 % w/w of inflorescence containing diet were given to diabetic Sprague Dawley rats for 45 days. Diabetes was induced in rats by alloxan administration (150 mg/kg bodyweight) intraperitoneally. Treatment with inflorescence significantly lowered blood glucose levels thereby preventing steep onset of hyperglycemia which was observed after alloxan administration. It maintained glucose tolerance and glycosylated hemoglobin close to the values observed in normal control rats. In addition, the levels of serum total cholesterol and triglycerides were also lowered. Antioxidant enzymes registered a decline in their activity in diabetic rats, thus revealing the damaging effects of free radicals generated due to alloxan administration. But their activities were reverted towards near-normal range in inflorescence-supplemented animals. Histopathological analysis of pancreas of diabetic rats showed altered morphology, which was restored to near-normal in inflorescence-treated rats. Oxidative damage in various tissues of diabetic rats as evidenced by marked elevation in the levels of thiobarbituric acid reactive substances was nullified by inflorescence treatment, indicating its antioxidant efficacy in resisting oxidative damage.

Keywords Alloxan · Antioxidant · Cocos nucifera inflorescence · Diabetes mellitus · Homeostasis · Oxidative stress

Introduction

Free radicals have been implicated in the pathogenesis of diabetes mellitus (DM). Hyperglycemia in DM may directly or indirectly contribute to excess formation of free radicals, which ultimately leads to oxidative stress [1]. Enhanced oxidative stress and changes in antioxidant capacity, observed in both clinical and experimental diabetes mellitus are thought to be the aetiology of chronic diabetic complications [2]. Implication of oxidative stress in the pathogenesis of diabetes is suggested not only by oxygen free-radical generation, but also due to nonenzymatic protein glycosylation, auto-oxidation of glucose [3], alteration in antioxidant enzymes [4] and lipid peroxide formation.

Alloxan is a cytotoxic glucose analogue, which selectively destroys pancreatic islets β-cells. Alloxan-induced DNA fragmentation in pancreatic islets and cell damage has been attributed to the production of toxic free radicals [5]. Thus, alloxan model was considered suitable for the study of experimental diabetes, in which free radicals might have a central role. Despite the introduction of hypoglycemic agents from natural and synthetic sources, diabetes and its secondary complications continue to be a major problem in the world. Current drugs used for the treatment of DM involve different mechanisms for bringing down blood glucose levels in normal or glycemic conditions but are associated with several side effects; hence there is need for effective, safe and better oral hypoglycemic agents [6]. Many indigenous Indian medicinal plants have been found to be useful to successfully manage diabetes [7].

Cocos nucifera (L.), belongs to the family Arecaceae, known as coconut palm, is attributed to possess various medicinal properties and culinary applications. Studies carried out using the coconut kernel protein and fiber are found to possess antidiabetic and hypoglycemic properties in experimental animals [8, 9]. In Cocos nucifera, the flowers
and flower bearing ramifications are together called inflorescence. In young inflorescence, the spikelets lie very close to the main axis (peduncle) and the whole is tightly packed. The watery sap that drips from immature inflorescence is used as a natural drink in tropical countries. In Ayurveda, Cocos nucifera inflorescence (CnI) is often used to cure backache. But the biochemical effect of CnI in controlling the complications of diabetes-induced oxidative stress has not been worked out. The present study is an attempt in this direction. In this study, the effect of dietary supplementation of inflorescence on diabetes and resultant oxidative stress has been investigated in alloxan induced diabetic rats.

Materials and methods

Collection and preparation of CnI

Young inflorescence of coconut palm (West Coast Tall variety) harvested from Kerala University campus was used for the study. The material was identified at Department of Botany in the University and a voucher specimen (No. KUBH 5795) was deposited in the herbarium. CnI was then pulverized using an electric laboratory blender. It was then dried at 55 °C in a hot air oven and the dry weight was taken and used for in vivo experiment.

Chemicals

Alloxan was purchased from Sigma Chemicals, St. Louis, USA. Kits for glucose, total cholesterol and triglycerides estimation were purchased from Agappe Diagnostics, Thane, India. All other chemicals used were of highest analytical grade possible.

Animals and experimental design

All the animal cares and procedures were according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. The experimental design and protocol used in this study was approved by Institutional Animal Ethics Committee (IAEC). One month old male Sprague Dawley rats (150–200 g BW) bred in our department animal house were used for the study. Diabetes was induced in 20 rats by injecting them with alloxan (150 mg/kg BW) dissolved in normal saline intraperitoneally after fasting the animals for 24 h. The rats were then kept on 5 % glucose solution for the next 24 h to prevent drug induced hypoglycemic phase. All the survived rats (65 %) were found to be diabetic after 72 h. Rats with a blood glucose level ≥250 mg/dL were considered to be diabetic.

All the animals were randomly divided into four groups (N0 6). Group I rats served as the normal control, while group II rats which were non-diabetic were given 20 % CnI (CnI control group). Group III rats served as the diabetic control. Diabetic rats in group IV were fed with 20 % CnI (treated group). In an earlier dose response study conducted in our laboratory, we used three doses (10, 20 and 40 % w/w of CnI) to compare and maximum effect was obtained from 20 % and that dose was selected for the study (unpublished result) (Table 1). Group I and group II animals received groundnut oil and glucosamine along with laboratory rat chow (VRK Nutritional Solutions, Pune, India.) (Table 2). Group II and IV animals received a diet which includes CnI, corn starch and casein along with laboratory rat chow. The different dietary components were given to each group so as to adjust the dietary intakes (% w/w) of all the four groups to be the same as shown in Table 3. The animals were housed individually in polypropylene cages in a room maintained at 25±10 °C with a 12 h light and 12 h dark cycle.

The experiment lasted for a period of 45 days. After the experimental period, animals were fasted overnight and sacrificed by intraperitoneal injection of thiopentone sodium (>40 mg/kg BW). Serum was separated from the blood samples collected through cardiac puncture and tissues were collected immediately and homogenized for various estimations.

Serum glucose and lipid levels

Estimation of serum glucose was done using a commercial kit based on glucose oxidase/peroxidase enzymatic method

| Table 1: Effect of 45 days treatment with different doses of Cocos nucifera inflorescence (CnI) on biochemical parameters in alloxan-induced diabetic rats |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Parameter       | Normal control  | Diabetic control| Diabetic treated with 10 % CnI for 45 days | Diabetic treated with 20 % CnI for 45 days | Diabetic treated with 40 % CnI for 45 days |
| Serum Glucose (mg/dl) | 86.76±8.3 | 242.28±23.2** | 151.12±14.4 | 123.8±11.8** | 129.67±12.4** |
| HbA1c (%)       | 4.87±0.36 | 8.08±0.77** | 6.53±0.62** | 6.04±0.57** | 6.54±0.77** |
| Total Hb (gm %) | 14.6±1.3 | 11.8±1.0* | 12.9±1.1 | 13.4±1.2** | 13.2±1.2** |

Values are mean±SD of six rats. *P<0.05 compared to normal control; **P<0.05 compared to diabetic control
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Table 2 Percentage dietary composition of dry CnI and laboratory rat chow.

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<td>Crude fiber</td>
<td>59.56%</td>
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Results

Effect of CnI on serum glucose, HbA1c and OGTT

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<td>Serum cholesterol</td>
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<td>Triglycerides</td>
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<td>Cardiac SOD</td>
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<tr>
<td>Renal SOD</td>
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<td>Hepatic CAT</td>
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<td>Hepatic TBARS</td>
<td>0.73±0.05</td>
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<td>Renal TBARS</td>
<td>2.85±0.24</td>
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The present study evaluates the biochemical effect of Cnl in alloxan-induced diabetes in experimental rats. The results demonstrated that Cnl significantly ameliorated the adverse influence of alloxan. Preliminary studies conducted by us revealed the non-toxic nature of Cnl. To the best of our knowledge; this is the first report utilizing Cnl as antidiabetic and antioxidative phytounutrient. Dietary supplementation of Cnl after alloxan treatment resulted in lower serum glucose levels, improved glucose tolerance and lipid profile as compared with rats administered alloxan alone. These changes were accompanied by a significant
The aldehydic product of lipid peroxidation, malonyldialdehyde (MDA) is more cytotoxic and stable than reactive oxygen species and reacts quickly with cellular constituents [25]. This in turn may contribute to the disruption of intracellular and membrane redox state of liver and pancreatic β-cells, hence disturbing glucose homeostasis. Dietary supplementation of CnI to diabetic rats reversed all these alterations to the basal levels. Moreover, the results of histopathological examination of the pancreas in experimental rats confirmed that CnI can inhibit β-cell shrinkage and reverse the reduction in the number of β-cells in diabetic rats, thereby maintaining the tissue integrity both in endocrine and exocrine pancreas.

Preliminary investigations revealed that CnI contains proteins, secondary metabolites like polyphenols, flavonoids and also soluble and insoluble fibers (unpublished result). It is suggested that the active principles from plant sources might act by several mechanisms such as stimulating insulin secretion, increasing repair or proliferation of β-cells, enhancing insulin sensitivity and increasing the antioxidative capability [26, 27]. CnI showed antidiabetic effects in experimental rats, which was evident from the biochemical and histopathological findings and that may be mediated via its antioxidative capability and β-cell repair. However, further studies are required to elucidate the detailed mechanism of action of CnI in this regard to develop it as a promising natural antidiabetic drug.

decrease in lipid peroxidation and increase in antioxidant status in the liver. These results are in accordance with those of other investigations using different phytonutrients [19, 20].

There are reports that hemoglobin undergoes non-enzymatic glycosylation in diabetes due to the presence of excess of blood glucose [21]. The significant decrease in HbAlc level in CnI-fed rats indicated the efficacy of CnI in better glycemic control. Previous studies suggest that, diabetes mellitus is often linked to abnormal lipid metabolism and the rise in serum glucose was accompanied by a marked increase in lipid profile [22]. The reduced levels of triglycerides and total cholesterol in our study imply that CnI may have an antihyperlipidemic effect in diabetic rats. Several studies showed that alloxan produces a decrease in the activity of the antioxidant enzymes during the development of alloxan-induced DM in liver and pancreas [23]. SOD plays a pivotal role in oxygen defense metabolism by intercepting and reducing superoxide to hydrogen peroxide, which is easily reduced to water by CAT. Therefore, the free radical scavenging activity of SOD is effective only when it is followed by the increased CAT activity [24]. Administration of alloxan produced a significant increase in aldehydic products of lipid peroxidation, indicating an increased hepatic oxidative stress which may also occur in other tissues in alloxan-treated rats.
Acknowledgement The financial assistance from KSCSTE, Trivandrum in the form of KSCSTE Fellowship is gratefully acknowledged.

Conflict of interest The authors declared no potential conflicts of interest.

References

1. El-Shenawy NS, Abdel-Nabi IM. Hypoglycemic effect of coleome droserifolia ethanolic leaf extract in experimental diabetes, and on non-enzymatic antioxidant, glycogen, thyroid hormone and insulin levels. Diabetol Croat. 2006;35:15.
Relationship between proinsulin and beta cell function in different states of glucose tolerance

Ping Yu 1 Qiang Li 1 Fengchen Liu 1 Yuqian Sun 1 Jinchao Zhang


Abstract To measure proinsulin concentrations in different states of glucose tolerance and examine the relationship between proinsulin and beta cell function. Serum true insulin (TI), proinsulin (PI), immunoreactive insulin (IRI), C peptide (CP) and blood glucose (BG) levels were measured in the fasting state and during an oral glucose tolerance test (OGTT) in 32 individuals with normal glucose tolerance (NGT), 42 individuals with impaired glucose tolerance (IGT), and 54 individuals with newly diagnosed type 2 diabetes mellitus. All participants were also subdivided into nonobese [body mass index (BMI) <25 kg/m²] and obese (BMI ≥25 kg/m²) subgroups. The levels of TI, PI, IRI and CP were higher in obese patients compared with the corresponding NGT subgroup, whereas there was no difference in PI/TI. The levels of IRI, PI and PI/TI were higher in nonobese patients with type 2 diabetes mellitus than in the corresponding NGT subgroup. The levels of PI and TI increased in obese patients with NGT and IGT, whereas PI/TI did not change. In contrast, PI increased but TI did not in subjects with newly diagnosed diabetes mellitus, which led to an increase in PI/TI, and a decrease in beta cell function. Therefore, PI and the PI/TI ratio could offer markers for beta cell dysfunction in DM.

Keywords True insulin · Proinsulin · Beta cell function · Glucose tolerance test

Introduction

Proinsulin (PI) is the precursor of insulin, and has similar physiological effects to insulin, albeit much weaker, being only 1/10–1/15 as effective as insulin [1–3]. Furthermore, the affinity of PI to the insulin receptor is only 1/20 of that of insulin [1–3]. Several recent studies have shown that the blood PI level increases in DM, but the duration and extent of this increase is not fully understood [4–6].

PI has been proposed as a marker for early beta cell dysfunction, and some studies have shown that the PI level is increased in obesity [7–9]. Therefore, do these findings mean that beta cell dysfunction is already apparent in obesity, even though the blood glucose level is normal, or is this finding due to insulin resistance? If insulin resistance is the trigger, the ratio of proinsulin and insulin would be higher in insulin-resistant subjects than in insulin-sensitive subjects. So the ratio leads to the diagnosis and treatment of insulin resistance in the early stage. In order to investigate the significance of the PI concentration, we carried out this study of individuals with different states of glucose tolerance, and calculated PI/TI to understand the significance of increased PI in obesity and DM.

Methods

Overall, 128 patients with varying states of glucose tolerance were randomly selected from our outpatients and underwent a routine physical examination. The protocol of our study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Clinical Research Ethics Committee at Harbin Medical University. Informed consent was obtained from the patients themselves before enrollment. Serum during an oral glucose tolerance
test (OGTT) were collected in 32 individuals with NGT (NGT group), 42 individuals with IGT (IGT group), 54 individuals with newly diagnosed type 2 DM. The study population was further subdivided into nonobese [body mass index (BMI) <25 kg/m²] and obese (BMI ≥25 kg/m²) subgroups. Blood pressure, height, weight, waist circumference and hip circumference were determined at the same time. Then true insulin (TI), immunoreactive insulin (IRI), PI, C peptide (CP) during OGTT were measured with kits (TI and PI: US Linco; IRI and CP: Atomic Energy Institute in China). The PT/TI ratio was calculated for each subject at each point during the OGTT.

All statistical data were analyzed using SPSS software package (SPSS Inc., Chicago, IL, USA). For continous variables with normal distributions, we used ANOVA, and for skewed distributions, we used the Wilcoxon rank sum test.

Results

General characteristics of subjects according to glucose tolerance

As shown in Table 1, age, sex ratio, waist/hip ratio, systolic blood pressure and diastolic blood pressure were not significantly different among the groups. BMI was not significantly different between the glucose tolerance states in the obese and nonobese subgroups. Cholesterol and triglyceride levels were significantly higher in subjects with DM than in those with NGT.

Results of the OGTT (Table 2)

Compared with the NGT group, IRI was significantly increased in the nonobese DM group at 0, 120 and 180 min after the glucose load (P0.048, 0.046, 0.038). In contrast, no significant difference was seen in the obese DM group compared with the NGT group at any time during the OGTT, CP increased significantly in the nonobese subjects with IGT at 120 min after the glucose load (P0.046), but no differences were seen in the other groups at any time. TI decreased significantly in the nonobese DM group at 60 min after the glucose load compared with the NGT group (P0.040), but no differences were seen in the other groups at any time. It should be noted that, at all times during the OGTT, PI was significantly increased in the obese and nonobese DM groups compared with the NGT group (all, P<0.05).

Compared with the corresponding nonobese subgroups, IRI, CP, TI and PI were all increased in the obese NGT and DM subgroups at all times during the OGTT. Moreover, fasting IRI and PI were significantly increased in obese subjects with IGT compared with nonobese subjects with IGT (P<0.05). Of particular interest, PI was significantly increased in all subgroups of obese subjects with DM, NGT or IGT compared with their nonobese counterparts (P<0.05).

The PI/TI ratio (Table 3)

The PI/TI ratio increased significantly at 0 and 60 min after the glucose load in obese and nonobese subjects with DM as compared with the respective subgroups of subjects with IGT or NGT (all, P<0.05). In contrast, there were no differences between the IGT and NGT groups.

Discussion

Under normal circumstances, 92 % of the PI in beta cells is processed to yield insulin and CP by two enzymes that cleave peptide chains. As a result, equimolar amounts of insulin and CP are released into the blood. Complete and partially processed proinsulin, also referred to as total proinsulin, accounts for 5–15 % of the normal adult’s circulating insulin. Intact PI, des-31, 32-proinsulin and des-64, 65-proinsulin account for approximately 30 %, 60 % and 6 %, respectively, of the circulating total proinsulin [1–3, 10–12].

Table 1 Subject characteristics

<table>
<thead>
<tr>
<th>Group</th>
<th>N (M/F)</th>
<th>Age (Y)</th>
<th>BMI (kg/m²)</th>
<th>Waist/hip ratio</th>
<th>SBP (mmHg)</th>
<th>DBP (mmHg)</th>
<th>Triglycerides (mmol/L)</th>
<th>Cholesterol (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGT-OB</td>
<td>16 (9/7)</td>
<td>40.3±15.8</td>
<td>30.20±4.92</td>
<td>0.88±0.07</td>
<td>124.1±27.1</td>
<td>85.0±14.5</td>
<td>1.80±0.94</td>
<td>4.05±1.54</td>
</tr>
<tr>
<td>NGT-NOB</td>
<td>18 (8/8)</td>
<td>39.1±10.1</td>
<td>21.77±2.23</td>
<td>0.82±0.05</td>
<td>119.5±22.9</td>
<td>79.1±16.4</td>
<td>1.12±0.22</td>
<td>4.61±0.89</td>
</tr>
<tr>
<td>IGT-OB</td>
<td>25 (14/11)</td>
<td>42.1±11.5</td>
<td>29.56±3.51</td>
<td>0.87±0.05</td>
<td>127.6±13.08</td>
<td>88.4±10.08</td>
<td>1.97±1.19</td>
<td>4.83±1.03</td>
</tr>
<tr>
<td>IGT-NOB</td>
<td>17 (8/9)</td>
<td>42.5±8.8</td>
<td>22.57±1.68</td>
<td>0.84±0.06</td>
<td>116.5±16.5</td>
<td>75.38±6.91</td>
<td>2.01±1.64</td>
<td>4.87±2.00</td>
</tr>
<tr>
<td>DM-OB</td>
<td>35 (20/15)</td>
<td>45.8±10.5</td>
<td>28.46±4.37</td>
<td>0.89±0.06</td>
<td>130.29±23.99</td>
<td>88.68±15.68</td>
<td>2.27±1.98*#</td>
<td>5.65±1.37*#</td>
</tr>
<tr>
<td>DM-NOB</td>
<td>35 (10/9)</td>
<td>49.6±6.6</td>
<td>23.30±1.04</td>
<td>0.86±0.07</td>
<td>123.68±14.22</td>
<td>79.74±10.20</td>
<td>2.70±1.62*#</td>
<td>5.68±1.71*#</td>
</tr>
</tbody>
</table>

Values are means ± standard deviation. *P<0.05 vs. the corresponding NGT subgroup; † P<0.05 vs. the corresponding NGT or IGT subgroups. BMI body mass index; SBP systolic blood pressure; DBP diastolic blood pressure; NGT normal glucose tolerance; OB obese; NOB nonobese; IGT impaired glucose tolerance; DM diabetes mellitus
<table>
<thead>
<tr>
<th>Group</th>
<th>0 min</th>
<th>60 min</th>
<th>120 min</th>
<th>180 min</th>
<th>0 min</th>
<th>60 min</th>
<th>120 min</th>
<th>180 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IRI (mIU/L)</td>
<td></td>
<td></td>
<td></td>
<td>CP (pmol/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NGT-OB</td>
<td>19.68±12.23*</td>
<td>86.00±65.09*</td>
<td>68.16±51.39*</td>
<td>34.07±23.00*</td>
<td>747.60±223.64*</td>
<td>2575.8±788.56</td>
<td>2385.3±1215.89*</td>
<td>1356.9±724.45*</td>
</tr>
<tr>
<td>NGT-NOB</td>
<td>9.99±3.44</td>
<td>47.27±22.22</td>
<td>25.84±13.59</td>
<td>13.80±12.27</td>
<td>524.70±215.85</td>
<td>2208.6±1034.74</td>
<td>1343.4±659.44</td>
<td>862.2±416.43</td>
</tr>
<tr>
<td>IGT-OB</td>
<td>18.38±10.89*</td>
<td>99.58±47.44</td>
<td>96.22±80.48</td>
<td>53.78±71.21</td>
<td>664.69±515.98</td>
<td>2651.0±1702.50</td>
<td>2266.8±1605.57</td>
<td>1357.8±1315.62</td>
</tr>
<tr>
<td>IGT-NOB</td>
<td>9.29±3.71</td>
<td>70.73±38.11</td>
<td>48.26±30.68#</td>
<td>20.45±16.99</td>
<td>615.07±421.17</td>
<td>2762.6±1062.42</td>
<td>2353.15±980.39#</td>
<td>1312.6±827.24</td>
</tr>
<tr>
<td>DM-OB</td>
<td>22.45±15.39*</td>
<td>65.72±47.80*</td>
<td>76.94±55.94*</td>
<td>49.74±40.57</td>
<td>996.64±507.45*</td>
<td>2054.5±1150.65*</td>
<td>2446.56±1308.25*</td>
<td>1998.0±1131.63</td>
</tr>
<tr>
<td>DM-NOB</td>
<td>16.94±12.69#</td>
<td>35.79±26.01</td>
<td>45.00±35.16#</td>
<td>35.70±32.17#</td>
<td>614.32±329.43</td>
<td>1527.43±650.18</td>
<td>1718.7±880.36</td>
<td>1414.36±800.09</td>
</tr>
<tr>
<td></td>
<td>TI (pmol/l)</td>
<td></td>
<td></td>
<td></td>
<td>PI (pmol/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NGT-OB</td>
<td>112.17±64.57*</td>
<td>550.89±432.59</td>
<td>354.01±261.32</td>
<td>172.18±111.77*</td>
<td>32.06±13.69*</td>
<td>71.53±29.45*</td>
<td>72.68±33.86*</td>
<td>63.01±31.97*</td>
</tr>
<tr>
<td>NGT-NOB</td>
<td>64.08±38.29</td>
<td>382.80±249.42</td>
<td>188.37±138.54</td>
<td>124.14±96.16</td>
<td>12.81±5.35</td>
<td>45.61±18.45</td>
<td>44.38±22.90</td>
<td>28.90±17.59</td>
</tr>
<tr>
<td>IGT-OB</td>
<td>131.93±93.30</td>
<td>889.49±594.52</td>
<td>593.09±501.32</td>
<td>134.86±124.28</td>
<td>37.2±33.37*</td>
<td>83.61±30.14*</td>
<td>82.72±29.78*</td>
<td>59.60±35.99</td>
</tr>
<tr>
<td>IGT-NOB</td>
<td>79.58±37.79</td>
<td>575.51±387.66</td>
<td>303.19±203.63</td>
<td>131.34±113.19</td>
<td>12.90±9.12</td>
<td>57.73±23.66</td>
<td>59.64±26.23</td>
<td>47.38±36.67</td>
</tr>
<tr>
<td>DM-OB</td>
<td>110.46±61.18</td>
<td>417.32±345.86*</td>
<td>398.06±312.57</td>
<td>271.31±218.29</td>
<td>59.72±28.148*#</td>
<td>87.62±21.63*#</td>
<td>92.92±22.90*#</td>
<td>87.22±21.95*#</td>
</tr>
<tr>
<td>DM-NOB</td>
<td>77.01±43.47</td>
<td>199.50±155.77##</td>
<td>240.45±186.56</td>
<td>182.12±120.42</td>
<td>41.63±25.49##</td>
<td>64.38±31.63#</td>
<td>74.05±31.20#</td>
<td>68.64±31.86#</td>
</tr>
</tbody>
</table>

Values are means ± standard deviation. * P < 0.05 vs. the corresponding NGT subgroup; ** P < 0.05 vs. the corresponding NGT or IGT subgroups. *P < 0.05 compared with the corresponding NOB subgroup with the same glucose tolerance state. IRI immunoreactive insulin; CP C-peptide; NGT normal glucose tolerance; OB obese; NOB nonobese; IGT impaired glucose tolerance; DM diabetes mellitus; TI true insulin; PI proinsulin

Table 3 Changes in the proinsulin/true insulin ratio during the oral glucose tolerance test

<table>
<thead>
<tr>
<th>Group</th>
<th>BG (mmol/l)</th>
<th></th>
<th></th>
<th></th>
<th>PI/TTI (%)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min</td>
<td>60 min</td>
<td>120 min</td>
<td>180 min</td>
<td>0 min</td>
<td>60 min</td>
<td>120 min</td>
<td>180 min</td>
</tr>
<tr>
<td>NGT-OB</td>
<td>4.24±0.38</td>
<td>5.85±0.83</td>
<td>5.74±0.98</td>
<td>4.31±0.98</td>
<td>31.92±14.73</td>
<td>17.70±9.78</td>
<td>26.23±14.80</td>
<td>40.61±20.08</td>
</tr>
<tr>
<td>NGT-NOB</td>
<td>4.77±0.46</td>
<td>6.31±1.11</td>
<td>5.65±1.35</td>
<td>4.72±0.86</td>
<td>20.13±9.75</td>
<td>14.60±6.32</td>
<td>28.54±13.25</td>
<td>37.25±21.52</td>
</tr>
<tr>
<td>IGT-OB</td>
<td>4.98±0.70#</td>
<td>9.93±1.86#</td>
<td>7.28±1.62#</td>
<td>4.71±1.42</td>
<td>25.86±19.64</td>
<td>13.11±10.11</td>
<td>20.96±20.51</td>
<td>40.64±27.73</td>
</tr>
<tr>
<td>IGT-NOB</td>
<td>5.32±0.68#</td>
<td>9.72±1.52#</td>
<td>7.19±1.50#</td>
<td>5.35±1.29</td>
<td>16.01±7.69</td>
<td>12.10±5.02</td>
<td>21.23±7.25</td>
<td>28.29±7.85</td>
</tr>
<tr>
<td>DM-OB</td>
<td>9.22±3.14##</td>
<td>18.64±4.51##</td>
<td>17.65±4.89##</td>
<td>13.21±5.54##</td>
<td>60.72±35.95##</td>
<td>36.28±28.34##</td>
<td>40.61±27.63</td>
<td>48.44±31.55</td>
</tr>
<tr>
<td>DM-NOB</td>
<td>10.91±3.85##</td>
<td>20.07±5.47##</td>
<td>21.34±5.77##</td>
<td>16.70±5.47##</td>
<td>62.83±38.90##</td>
<td>46.77±32.33##</td>
<td>51.39±37.62##</td>
<td>64.23±46.89##</td>
</tr>
</tbody>
</table>

Values are means ± standard deviation. * P < 0.05 vs. the corresponding NGT subgroup; ** P < 0.05 vs. the corresponding NGT or IGT subgroups. BG blood glucose; PI proinsulin; TI true insulin; NGT normal glucose tolerance; OB obese; NOB nonobese; IGT impaired glucose tolerance; DM diabetes mellitus
Recent studies have revealed that the levels of PI, particularly des-31, 32-proinsulin, are elevated in patients with DM or obesity, and after pancreatic resection [13, 14].

This study showed that PI secretion was significantly increased in obese people than in nonobese people with normal glucose tolerance (P < 0.05) and that the secretion of TI increased, without significant changes in the PI/TI ratio. Therefore, we suggest that obesity-induced insulin resistance may result in greater synthesis and secretion of TI from beta cells to meet the needs of the body, with corresponding increases in PI, the precursor of TI. Therefore, the increased synthesis and secretion of PI was a result of increased synthesis and secretion of TI because of insulin resistance in obesity, rather than representing a decline in beta cell function [15].

We also found that in patients with newly diagnosed DM, the increases in PI (Table 2) were associated with increases in PI/TI (Table 3). We believed this result reflects beta cell dysfunction because PI secretion increased whereas TI did not in obese and nonobese subjects with newly diagnosed DM. Even the TI level 60 min after the glucose load in subjects with DM was significantly lower than that in nonobese subjects with NGT or IGT (Table 2). These results may be due to impaired processing of PI to form TI in beta cell in DM, resulting in incomplete cleavage of PI. In this study, the changes in beta cell function do not seem to be significant based on changes in TI or CP. Even in subjects with DM, the levels of TI and CP were not significantly decreased during the OGTT except at 60 min after the glucose load in nonobese subjects with DM. The early changes in pancreatic islet cells can only be detected when PI is determined and these changes were different from those of insulin-resistant individuals. Indeed, the latter had increased PI levels, which were accompanied by increased TI levels. This suggests that PI/TI can be used as a marker for early beta cell dysfunction. The increase in PI/TI occurred in association with impairments in beta cell secretory function in the initial stage of DM, resulting in elevated blood glucose levels in patients with DM [16–18].

There are many methods that can be used to evaluate beta cell function in the clinic, and most of them involve calculation of an index of islet cells by determining the amount of insulin secreted after a glucose load. Unfortunately, these methods are unable to directly detect changes in beta cell function, except for obvious changes that occur in the insulin or glucose levels. In this study, we assessed changes in beta cell function at an early stage of pathogenesis of DM by calculating PI/TI and measuring PI directly.

To conclude, the levels of PI and TI were increased in insulin-resistant, obese subjects with NGT and IGT. However, because the ratio of PI/TI was unchanged, beta cell function can be assumed to be normal. However, in newly diagnosed DM, PI increased without corresponding increase in TI, which resulted in a marked increase in PI/TI. This indicates marked reduction in beta cell function.

Acknowledgments This work was supported by a grant from the Science and Technique Foundation of Heilongjiang Province of China (GC02C154) and by the Science and Technology Planning Project of the Educational Commission of Heilongjiang Province, China (1051065).

Conflicts of interest None

References


Screening for Cushing’s syndrome in obese type 2 diabetic patients and the predictive factors on the degree of serum cortisol suppression

Meral Mert, Mustafa Temizel, Selahattin Erol, Yucel Arman, Kadem Arslan, Feride Alakus, Ebru Ayozturk Velioglu


Abstract  The aim of this study was to examine the frequency of Cushing’s syndrome (CS) in obese type 2 diabetic patients devoid of specific clinical symptoms of Cushing’s syndrome. A total of 148 obese (BMI ≥ 30 kg/m²) type 2 diabetic patients (113 female, 35 male) were included in the study. An overnight 1 mg dexamethasone suppression test (DST) was performed on all patients. Suppression of serum cortisol to < 1.8 µg/dL after administration of 1 mg dexamethasone was considered normal suppression. Low dose dexamethasone suppression test was performed on the patients who had serum cortisol level over 1.8 µg/dL after overnight 1 mg DST. Regression analysis was applied to determine the effective factors on suppression of serum cortisol. Mean age, BMI and HbA1c levels respectively were 50.82 ± 8.50 year, 31.78 ± 4.66 kg/m², %8.96 ± 2.42 in males and 54.15 ± 10.348 year, 34.32 ± 5.71 kg/m², % 8.18 ± 2.06 in females. Serum cortisol level was found 1.64 ± 5.62 µg/dl after overnight DST. A total of 9 (6.2 %) patients had non-suppressible overnight DST. Only four (2.6 %) of these patients were diagnosed with Cushing Syndrome after low-dose DST. Diagnosis was confirmed pathologically. Etiologic reasons for Cushing’s syndrome were pituitary microadenoma (2 patients) and adrenocortical adenoma (2 patients). Age and duration of diabetes was found to be related to the degree of suppression. Cushing’s syndrome should be investigated in high-risk groups like uncontrolled diabetes, obesity. All the related factors on the degree of suppression must be considered for the final diagnosis.

Keywords  Cushing’s syndrome · Diabetes mellitus · Obesity · Cortisol

Introduction

Diabetes mellitus (DM) is a hyperglycemic disease that results in anomalies in carbohydrate, protein and fat metabolism [1]. More than 80 % of patients with type 2 DM are already overweight [2]. Obesity is the most common metabolic problem in industrialized countries. In the USA, 32 % of adults are classified as overweight, and 22.5 % of these individuals are obese [3].

Obesity can coexist with diverse physical disorders, and physicians should first focus on the differential diagnosis of possible underlying disorders before initiating any management procedure.

Obesity is a frequent clinical sign of Cushing’s Syndrome. Adipose tissues in CS patients typically have a central distribution [4]. Diagnosis of CS is primarily based on the signs and symptoms of the disorder. Nevertheless, a
significant number of CS patients present only with simple obesity [5, 6] or with type 2 diabetes mellitus and poor glycemic control [7–9]. Metabolic syndrome may also indicate the presence of CS. Patients without specific clinical features of CS have been referred to as sub-clinical Cushing’s syndrome patients. The diagnosis of CS poses a considerable challenge to the physician [7]. False positive results after dexamethasone suppression tests may be found due to occult Cushing’s Syndrome. Treatment of occult CS may have more beneficial effects than treatment of diabetes mellitus and cardiovascular risk factors [8].

We aimed to determine the frequency of CS in type 2 obese diabetic patients and the effective factors on the degree of serum cortisol suppression.

**Patients and methods**

This study included 148 obese type 2 diabetic patients (113 females, mean age: 54.15±11.34 year; 35 males, mean age: 50.82±8.50 year, body mass index (BMI) >30 kg/m²) who were followed and treated at the Okmeydani Teaching Hospital diabetes outpatient clinic. All patients had metformin therapy, 113 of 148 patients were receiving oral anti-diabetic therapy and 35 patients were on insulin therapy (31 patients being treated with analog insulin mixtures twice daily, 32.49±5.89 units/day; 4 patients being treated with intensive insulin therapy four times daily, 41.38±8.27 units/day). Written informed consent was obtained from all patients before commencement of the study. Review of patient medical history was used to exclude subjects in which the dexamethasone suppression test could be altered either by medication (including exogenous glucocorticoid intake) or by other factors known to influence the test (including drug use, alcoholism, obvious depression, or pregnancy). None of the patients had nephropathy (creatinine clearance <30 ml/min), acute illness, or sleep disorders.

All patients in our study had obesity. Although the patients had obesity, careful examinations did not reveal hirsutism, buffalo hump, easy bruising, or any other manifestations suggestive of CS.

Clinical characteristics including BMI and anthropometric measurements were recorded. Morning blood samples following fasting (12 h) were drawn from an antecubital vein for determination of the concentrations of cortisol (N: 5–28 μg/dL), glucose, HbA1c, serum lipids (total cholesterol, LDL, HDL, VLDL-cholesterol, and triglyceride).

All patients subsequently underwent a 1-mg overnight dexamethasone suppression test (DST). Suppression of serum cortisol to <1.8 μg/dL after dexamethasone administration was considered normal suppression. Measurement of serum ACTH levels and an 2-mg 2 days DST were performed as second-step investigations in all patients who failed to achieve serum cortisol suppression <1.8 μg/dL after administration of 1 mg DST. Complementary imaging studies, magnetic resonance imaging (MRI) of the sella, cavernous sinus sampling, and abdominal computed tomography (CT) were performed when the results of the second-step evaluations were consistent with ACTH-dependent or ACT-independent CS.

Cortisol and ACTH were assessed by radioimmunooassay (RIA) using a Packard Riasvar gamma counter (Perkin-Elmer, Waltham, MA, USA) and commercial assay kits. Other biochemical laboratory tests were measured with an Olympus AU 600 autoanalyser (Olympus, Tokyo, Japan).

Calculations were done with SPSS software (version 11.5; SPSS Inc, Chicago, IL, USA), and differences in the values of the variables between the groups were evaluated by the Mann–Whitney U test. The degree of the correlation between parameters was evaluated by regression analysis. A value of P<0.05 was considered to be statistically significant.

**Results**

We analyzed a series of patients presenting with obesity for the presence of sub-clinical CS. The clinical characteristics and laboratory findings for these patients are summarized in Table 1.

A total of 9 (6.2 %) patients had impaired overnight DST (failure to suppress cortisol <1.8 μg/dL). Only four (2.6 %) of these patients were diagnosed with Cushing’s Syndrome after low-dose DST. Diagnosis was confirmed pathologically. Etiologic reasons for Cushing’s syndrome were pituitary microadenoma (2 patients) and adrenocortical adenoma (2 patients). Age and duration of diabetes was found to be related to the degree of suppression.

Complementary imaging studies were performed on these 4 CS patients, which revealed pituitary microadenoma in 2 patients and adrenal adenoma in 2 patients.

Age and duration of diabetes were found effective on the degree of serum cortisol suppression (suppression’s degree increased as the age or duration of diabetes increased) but gender, BMI, hypertension, hyperlipidemia and HbA1c were found to be ineffective in regression analysis (Table 2).

**Discussion**

Cushing’s syndrome is not an uncommon disease. Mortality and morbidity rate are high and clinical signs and symptoms are present in many clinical situations but not necessarily so. For this reason screening of high-risk groups is of great importance.

CS screening in simple obesity is usually not performed unless the patient has other clinical features of CS, such as poorly controlled hypertension, diabetes, osteoporosis with
rapid progression, or hypokalemia that is unresponsive to the therapy. Delayed identification of CS in obese patients results in the progression of CS, with the risk of irreversible complications. Some patients with type 2 diabetes and poor glycemic control may also have CS, and these patients should be screened for CS. Recent studies have provided a rationale for the systemic screening of CS in patients with type 2 diabetes [9, 10]. It has been well documented since the 1960s that patients with CS typically suffer from hyperinsulinemia, insulin resistance, and type 2 diabetes [11, 12].

Table 1: Findings in the study group

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>50.82±8.50</td>
</tr>
<tr>
<td>Women</td>
<td>54.15±10.34</td>
</tr>
<tr>
<td>Total</td>
<td>53.25±10.01</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>31.78±4.6</td>
</tr>
<tr>
<td>Women</td>
<td>34.32±5.71</td>
</tr>
<tr>
<td>Total</td>
<td>33.77±5.55</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>8.96±2.42</td>
</tr>
<tr>
<td>Women</td>
<td>8.18±2.06</td>
</tr>
<tr>
<td>Total</td>
<td>8.38±2.71</td>
</tr>
<tr>
<td>Duration of diabetes (year)</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>4.86±4.23</td>
</tr>
<tr>
<td>Women</td>
<td>5.91±4.67</td>
</tr>
<tr>
<td>Total</td>
<td>5.63±4.56</td>
</tr>
<tr>
<td>Basal cortisol (µg/dL)</td>
<td>11.24±4.52</td>
</tr>
<tr>
<td>Serum cortisol after overnight</td>
<td>1.64±5.62</td>
</tr>
<tr>
<td>DST (µg/dL)</td>
<td>199.85±47.75</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>192.2±130.63</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>48.25±3.75</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>119.10±37.56</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>37.98±24.85</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td></td>
</tr>
</tbody>
</table>

Catargi et al screened 200 overweight inpatients with type 2 diabetes with poor glycemic control (HbA1c > 8%). None displayed the specific clinical features of CS in addition to their poorly-controlled type 2 diabetes. A total of 52 (26%) patients had abnormal 1-mg DST (failure to suppress cortisol < 2.1 µg/dL). Of these, 17 (8.5%) displayed at least one additional biological abnormality of the hypothalamic-pituitary-adrenal axis. Definitive occult CS was identified in 4 patients (2%), with 3 (1.5%) having Cushing’s disease and 1 (0.5%) with surgically proven adrenal adenoma. However, definitive diagnosis of CS in the remaining seven patients (3.5%) required further investigation. These seven patients, therefore, had probable adrenal-dependent CS but did not undergo surgery. Six months after treatment for CS, a mean 5.5% reduction in body weight and a mean 2.5% reduction in HbA1c were reported in four patients. Oral antidiabetic medications were resumed in one patient, and the daily insulin dose was dramatically reduced in another. This study highlights the need to perform prospective CS screening on overweight, poorly controlled type 2 diabetic patients [7].

Leibovitz et al. assessed the prevalence of pre-clinical CS among obese patients with uncontrolled diabetes. In this study, 90 obese poorly controlled diabetic patients (BMI > 25 kg/m² and HbA1c > 9%), underwent an overnight 1 mg DST. In patients with non-suppressible cortisol levels (>140 nmol/l), Liddle’s 2 and 8 mg dexamethasone suppression tests and imaging studies were performed, 4 patients failed to suppress plasma cortisol (< 140 nmol/l). In one patient the diagnosis of CS was not confirmed by a standard Liddle’s test and was therefore considered false positive. In the other 3, the diagnosis of CS was confirmed (3%) [13].

Also the medical records of 63 patients with endogenous CS were reviewed in this study. In this retrospective analysis, 11 (17.5%) had diabetes and 2 (3.2%) lacked the classic physical characteristics of the syndrome.

Tiryakioğlu et al. examined the frequency of Cushing’s syndrome (CS) in obese patients devoid of specific clinical symptoms of Cushing’s syndrome A total of 150 obese patients (129 female, 21 male; mean age 44.41±13.34 year; mean BMI 35.76±7.13) were included in the study. Cushing’s syndrome was diagnosed in 14 of the 150 patients (9.33%) [14]. One of the similar studies which was performed by Baid et al demonstrated that the results of screening tests specific to CS may be falsely abnormal in overweight and obese populations [15].

Abromof et al. demonstrated that overnight dexamethasone suppression test is a valid screening test for CS in obese patients. Moreover, they found 2.3% false positive rate for the overnight dexamethasone suppression test while 3 microg/dL was chosen as the cut-off point for the suppression [16]. Sahin et al documented that 8% and 2% false positivity rate for the screening of CS in simple obese patients [17].
In this study we documented 4 cases of CS in a sample of 148 obese diabetic patients (2.6%). This result is lower than other studies. The characteristics of study population may have influenced the result. Age and duration of diabetes were found to be related and gender, BMI, hypertension, hyperlipidemia, HbA1c were found to be unrelated to the degree of serum cortisol suppression. For this reason, related factors and false positivity rate should be considered for the evaluation of the results.

Conflict of interest  None

References

Estimate of the diabetic retinopathy hazard rates in type 2 diabetic patients with current status data
Mohsen Askarishahi 1 Ebrahim Hajizadeh 1 Mohammad Afkhami-Ardekan 1 Masoud Reza Manaviat

Abstract Diabetes Retinopathy is an important microvascular complication of diabetes mellitus and estimation of the retinopathy prevalence from cross sectional data is very important. This study is to estimate the hazard rate of diabetic retinopathy when only cross sectional data measuring diabetes prevalence is available. A total of 459 type 2 diabetic patients referred to an Ophthalmology clinic from May to December 2008 were studied. The severity of diabetic retinopathy was graded as per Early Treatment Diabetic Retinopathy Study (ETDRS). Factors associated with occurrence of retinopathy were assessed by the Cox’s proportional hazard model for current status data. Multivariate analysis showed that body mass index, gender, smoking status and family history of diabetes were more independent predictions of diabetic retinopathy, while method of treatment, history of hypertension and duration of diabetes were all significantly associated with the occurrence of diabetic retinopathy. Compared with oral treatment group, those on insulin were more likely to be associated with diabetic retinopathy. From the cross sectional data, it is concluded that diabetic retinopathy hazard rate are less in patients with hypertension and more in patients using insulin and those with long duration of diabetes.

Keywords Diabetes retinopathy · Diabetes type 2 · Proportional hazard · Current status data

Introduction
Diabetic retinopathy (DR) is an important and common microvascular complication of diabetes mellitus. It affects 30%–50% of all diabetic patients and represents the main cause of legal blindness in patients aged 20–74 years in developed countries [1]. The Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) showed that 1.6% of type 2 diabetic patients were legally blind [2]. Up to 21% of patients with type 2 diabetes had DR at the time of diagnosis of diabetes [3]. The high prevalence and severity of DR suggest the need for screening program to recognize it as early as possible; this recommendation becomes even more important since DR may be asymptomatic even in its more advanced stages [4]. Therefore, the occurrence of DR over time among type 2 diabetic patients is an important public health parameter. However, in practice the DR hazard rate is difficult and expensive to estimate since it requires following a cohort of individuals over time. Estimation of DR prevalence is much less costly and can be done by a cross-sectional study. This problem prompted us to apply regression of current status data in order to identify patients at risk to develop DR when only DR cross-sectional data have been collected over an extended period of time. By determining the potential predictors of DR, we can identify at risk patients so that appropriate intervention can be undertaken.

Subjects and methods
This study included a total of 459 type 2 diabetic patients referred to the Ophthalmology Clinic of Yazd Diabetes Research Center, Shahid Sadoughi University of Medical
Sciences, from May to December 2008. An inclusion criterion was only type 2 diabetes mellitus diagnosis and exclusion criterion was end-stage renal disease. The study was approved by the Medical Ethics Committee of Shahid Sadoughi University of Medical Sciences and Health Services of Yazd and informed consent was obtained from all subjects. All patients underwent an ophthalmic examination by an ophthalmologist including slitlamp biomicroscopy and indirect ophthalmoscopy with dilated pupils. An ophthalmologist graded according to the Early Treatment Diabetic Retinopathy Study (ETDRS). DR was graded as no retinopathy (NO DR), Mild NPDR (Mild Non Proliferative Diabetic Retinopathy), Moderate NPDR (Moderate Non Proliferative Diabetic Retinopathy), Severe NPDR (Severe Non Proliferative Diabetic Retinopathy) and proliferative diabetic retinopathy (PDR). Then the patients were divided into two groups based on the presence or absence of DR. Information including age at the monitoring time (year), gender, duration of diabetes (year), smoking status (yes, no), history of family diabetes (yes, no) and the mode of treatment (oral, insulin) were collected. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared.

Statistical analysis

Statistical analyses were carried out by SPSS (version 17.0) and using the intcox package of R software (version 2.12.2) [5]. Characteristics of the study population are described using means for continuous variables and percentages for categorical variables. T-tests were used for continuous variables, and \( \chi^2 \) test for categorical variables. In order to identify the effect of other variables in hazard rate for DR, the data were analyzed using Cox’s proportional hazard model for current status data [6]. Current status data arise when the exact timing of an event is unobserved, and the only available information is whether or not the event has occurred. Such data commonly occur in many fields of epidemiology, biomedicine, demography and econometrics [7]. In our study, individuals who were diagnosed with DR (DR presence) were left-censored since we know only that the DR onset time is greater than the monitoring time. Individuals who were not diagnosed with DR (DR absence) were right-censored since we know only that the DR onset time is greater than the monitoring time. The DR onset time is not observed directly. Univariate and multivariate Cox’s proportional hazard model were used to determine the ability of each variable to estimate the hazard rate of DR. In the univariate analyses, we first considered each variable separately and those variables that had a P-value of less than 0.1 were considered as statistically significant and then these variables were further included into the multivariate proportional hazard model. For estimation of the hazard rate of DR and 95% confidence intervals and P values were calculated. A P-value of less than 0.05 was considered to indicate a significant difference. Univariate hazard curves were generated by the cox’s proportional hazard method.

Results

The study population included 459 type 2 diabetic patients. DR was diagnosed in 187 patients, giving a prevalence of 40.7%. Of these 187 patients, 171 (36.3%) had NPDR and 16 (3.5%) had PDR. Table 1 shows the frequency of patients according to type of retinopathy at the monitoring time.

In this study the patients were divided into two groups based on the presence or absence of DR and then the predictive factors were assessed. Comparing the characteristics between patients with DR and patients without DR at the monitoring time (Table 2), those with DR were younger (55.0 vs. 53.8 years, P<0.05), and had a longer duration of diabetes (14.4 vs. 7.9 years, P<0.05), were more likely to be females (64.2% vs. 59.2%, P<0.05), had lower BMI (26.9 vs. 27.9 kg/m², P<0.05), had higher history of hypertension (42% vs. 28%, P<0.05), had higher family history of diabetes (52% vs. 49%, P<0.05), and were less smokers (10% vs. 12%, P>0.05). Furthermore, more patients without DR were on oral treatment (96% vs. 79% P<0.05).

A univariate cox proportional hazard analysis was conducted to determine the factors that are associated with the hazard rate of DR (Table 3). Significant predictors in the univariate models were diabetes duration, method of treatment, and history of hypertension. This table shows that for every year increase in the duration of diabetes the hazard rate for DR increases by 1.05 times and patients who had been treated with insulin had a 110% higher risk of DR compared with those on oral treatment (HR 2.10; 95% CI, 1.474–2.992). Patients with a history of hypertension had a 63% higher risk of DR compared with patients without it. Figure 1 presents the estimates of the hazard functions of DR for the three variables, which were significantly associated with hazard rate of DR under univariate model. According to Fig. 1a the rising trend of the hazard rate of DR indicates that duration of diabetes is probably the strongest predictor for development of retinopathy. It can be seen

<table>
<thead>
<tr>
<th>Type of retinopathy</th>
<th>N</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No diabetic retinopathy</td>
<td>272</td>
<td>(59.3)</td>
</tr>
<tr>
<td>Mild non-proliferative diabetic retinopathy</td>
<td>99</td>
<td>(21.6)</td>
</tr>
<tr>
<td>Moderate non-proliferative diabetic retinopathy</td>
<td>62</td>
<td>(13.5)</td>
</tr>
<tr>
<td>Severe non-proliferative diabetic retinopathy</td>
<td>10</td>
<td>(2.2)</td>
</tr>
<tr>
<td>Proliferative diabetic retinopathy</td>
<td>16</td>
<td>(3.5)</td>
</tr>
<tr>
<td>Total</td>
<td>459</td>
<td>(100)</td>
</tr>
</tbody>
</table>

Table 1 Frequency of patients according to retinopathy grade
Table 2 Comparison of characteristics of the patients with the presence of diabetic retinopathy vs. patients with the absence of diabetic retinopathy

<table>
<thead>
<tr>
<th></th>
<th>Diabetic retinopathy</th>
<th></th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Presence (N=187)</td>
<td>Absence (N=272)</td>
<td>Total (N=459)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>55.0±9.9</td>
<td>53.8±10.2</td>
<td>55.0±9.9</td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>14.4±6.2</td>
<td>7.9±5.0</td>
<td>10.5±6.4</td>
</tr>
<tr>
<td>BMI* (kg/m2)</td>
<td>26.9±3.9</td>
<td>27.9±4.7</td>
<td>27.5±4.4</td>
</tr>
<tr>
<td>Gender (females)</td>
<td>120 (64.2)</td>
<td>161 (59.2)</td>
<td>281 (56.8)</td>
</tr>
<tr>
<td>Smoking status (yes)</td>
<td>18 (10)</td>
<td>33 (12)</td>
<td>51 (11.1)</td>
</tr>
<tr>
<td>History of hypertension (yes)</td>
<td>79 (42)</td>
<td>77 (28)</td>
<td>156 (34.0)</td>
</tr>
<tr>
<td>Family history of diabetes (yes)</td>
<td>97 (52)</td>
<td>134 (49)</td>
<td>231 (50.3)</td>
</tr>
</tbody>
</table>

Data are expressed as N (%) or mean ± SD

*BMI body mass index

NS not significant

*P based on T-test (continuous) comparing differences and x² (categorical)

from Fig. 1b that the separate estimates seem to be reasonably close to each other in younger ages (30–50 years of age) and the difference between DR hazard rates mainly occurs in older ages (above 50 years of age) for the patients in the two methods of treatment. Both patients without history of hypertension and known hypertension showed that the trend in the risk of DR increased with age in monitoring time. The risk of DR in the patients with no history of hypertension was higher compared to the patients with history of hypertension (Fig. 1c). Gender, BMI, smoking status and family history of diabetes had no statistically significant effect on hazard rate of DR. The hazard rate of DR was significantly higher in males (HR 1.077; 95 % CI, 0.798–1.454, P>0.05) compared to females. The hazard rate for DR was lower in smokers than in non-smokers (HR 0.812; 95 % CI, 0.499–1.321, P>0.05) and family history of diabetes mildly increased the hazard rate of DR (HR 1.179, 95 % CI 1.0085–1.571, P>0.05). We did not find any association between BMI and the occurrence of DR but a protective effect was shown in subjects with BMI≥30 compared to those with BMI<25. However the hazard rate of DR was notably lower for obese individuals (HR0.839, 95 % CI0.548–1.2870, P>0.05). After univariate Cox proportional hazard evaluation, 3 factors including diabetes duration, method of treatment and history of hypertension entered multivariate analysis. The results are shown in Table 1. The influence of hypertension and method of treatment on occurrence of DR reached a very significant level (P<0.001). Patients with a history of hypertension had a lower risk for DR (HR 1.69; 95 % CI, 1.474–2.992).

**Discussion**

In this study the prevalence of DR in Yazd in 2008 was estimated to be 40.7 %. The prevalence of DR in our study was higher than that of another local study conducted by Javadi et al. which was 37 % in Tehran province [8]. This difference may be due to higher prevalence of type 2 diabetest in Yazd compared with other provinces of Iran [9]. Most of the Asian studies indicated a much lower prevalence of DR, some of these studies used clinical examinations such

<table>
<thead>
<tr>
<th>Variables</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>CI 95 %</td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI* (BMI≥25, vs)</td>
<td>1.05</td>
<td>1.032–1.072</td>
</tr>
<tr>
<td>25&lt;BMI≤30</td>
<td>1.115</td>
<td>0.802–1.155</td>
</tr>
<tr>
<td>BMI≥30</td>
<td>0.839</td>
<td>0.548–1.287</td>
</tr>
<tr>
<td>Gender (male vs female)</td>
<td>1.077</td>
<td>0.798–1.454</td>
</tr>
<tr>
<td>Method of treatment (Insulin vs oral)</td>
<td>2.100</td>
<td>1.474–2.992</td>
</tr>
<tr>
<td>Smoking status (yes vs no)</td>
<td>0.81</td>
<td>0.499–1.321</td>
</tr>
<tr>
<td>History of hypertension (yes vs no)</td>
<td>1.632</td>
<td>1.182–2.253</td>
</tr>
<tr>
<td>Family history of diabetes (yes vs no)</td>
<td>1.179</td>
<td>0.885–1.571</td>
</tr>
</tbody>
</table>

HR hazard rate; CI confidence interval
Fig. 1  a Cumulative hazard rate of DR in diabetic patients based on mean of diabetes duration. b Cumulative hazard rate of DR in diabetic patients based on risk stratification according to differences in method of treatment. c Cumulative hazard rate of DR in diabetic patients based on risk stratification according to differences in history of hypertension.
as reports from Pakistan and India, which demonstrated 17.5 % and 26.2 % DR prevalence, respectively [10, 11]. These differences may be due to variations in screening program, sample size, limitation in compensation of confounders and the diagnostic method (imaging vs. clinical). There are multiple risk factors that can affect the hazard rate of DR. In this study, as shown in other studies, women compared to men, had a slightly higher rate of DR. However, in the Cox regression analysis, this risk disappeared and presence of diabetic retinopathy was independent of gender. This was in contrast to studies on other populations in which a male preponderance had been reported, such as in the United Kingdom Prospective Diabetes Study (UKPDS) Kohner et al. [12], and the Los Angeles Latino Eye Study [13]. In Wisconsin study [14] men had a slightly higher rate of retinopathy and this finding is in agreement with ours. The reason for the discrepancy is not yet clear.

There is controversy regarding the role that smoking plays in the risk of DR. Most studies found no significant association between smoking and DR. Hoorn’s study showed that cigarette smokers and ex-smokers had higher but non-significant odds ratios compared with those of individuals who had never smoked [15, 16]. In contrast, the UKPDS demonstrated that current smoking status was associated with a reduced incidence and progression of DR compared with those who had never smoked, with a relative risk of 0.63 and 0.50, respectively. In our study, fewer subjects had a history of smoking, compared with the UKPDS (11.1 % vs. 66 %) [17]. We found that a history of smoking was negatively associated with the hazard rate of DR in our population and the hazard rate of DR increased in non-smokers, although this was not statistically significant.

The association of BMI and DR is ambiguous. Most studies have reported positive associations between high BMI or obesity with DR [18, 19]. But others have reported contradictory results, in which higher BMI levels may be protective of DR [2, 20, 21]. Dowse et al., and Chaturvedi and Fuller reported that decreasing BMI is associated with a higher prevalence of DR [18, 22]. In our study there was an inverse relationship between BMI and DR, in which subjects with the highest levels of BMI were less likely to have DR, but this result was not statistically significant. Our results are in agreement with those of the latter studies.

Family history of type 2 diabetes has been associated with both DR and diabetic nephropathy [2]. In the present study we did not observe any association between family history of diabetes and the risk of DR. In agreement with our results Chen et al., showed that family history of diabetes had no significant association with retinopathy [23]. As the retinopathy at the early stages does not seem to progress, but patients with a family history of diabetes are recommended to have their blood glucose regularly checked since in other studies such individuals have shown to be at risk of developing diabetes.

Many studies have demonstrated that duration of diabetes contributes to the pathogenesis of DR. In our study diabetic patients with DR had a longer duration of diabetes (14.4 vs. 7.9 years; P < 0.0001). The hazard rate of DR increased with duration of diabetes. We have observed that for every one year increase in the duration of diabetes the risk for DR increases by 1.048 times. In the study conducted by Dandona et al. [24] on type 2 diabetes, it is demonstrated that 87.5 % of those with duration of diabetes >15 years had DR compared with 18.9 % of those who had <15 years duration. Wisconsin Epidemiologic Study of Diabetic Retinopathy reported that for every five year increase in duration of diabetes, the risk for DR increased by 1.89 times [20]. The duration of diabetes is probably the strongest predictor for development and progression of DR.

In our study, there was a clear gradient hazard rate of DR according to diabetes mellitus treatment type. The lowest rate was seen in the group with oral treatment and the highest rate existed among insulin users. Compared with the oral treatment group, persons using insulin therapy were 1.81 times (95 % CI, 1.239–2.627) more likely to have DR. The use of insulin has also been shown to be associated with a higher incidence of DR development in some studies [25] and the Wisconsin Epidemiologic Study of Diabetic Retinopathy reported a 10-year DR incidence of 67 % for non-insulin users and 79 % for insulin users [2]. According to the findings of a four-year cohort study, conducted by Manavat et al., the risk of DR in insulin group was 1.55 times more than oral hypoglycemic agent [26].

Hypertension is a major risk factor for cardiovascular events, such as myocardial infarction and stroke, as well as for microvascular complications, such as retinopathy and nephropathy [27]. It is 1.5 to 3 times more common in patients with diabetes than in non-diabetics [28]. Our univariate and multivariate analysis indicated that history of hypertension decreases the risk of DR. On the other hand, this study apparently revealed that history of hypertension has a protective effect against occurrence of DR. Some study reported that hypertension markedly increases the risk of microvascular complications, such as nephropathy and retinopathy [29]. The authors believe that this result is probably due to the effect of anti-hypertensive drugs on cardiovascular events in diabetic patients who were under treatment with these drugs. In further examination, we found that 156 of patients had history of hypertension, 133 of whom were under treatment with anti-hypertensive drugs. In agreement with our results, the U.K. Study (UKPDS) demonstrated that over a nine-year follow-up tighter blood pressure control led to a decreased risk in the progression of retinopathy [30]. In another study over a five-year follow-up period, Schrier et al reported that intensive blood pressure control decreased the progression of DR [31].
Controlling hypertension is the key to reduce cardiovascular risk in patients with diabetes.

Acknowledgments The authors would like to thank all the patient participants in this study and the staff of Yazd Diabetes Research Center who helped us in collecting data.

Conflict of interest None declared.

References

Original Article

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Evaluation of plasma fibrinogen levels in type 2 diabetes mellitus
K. N. Pannag Desai & M. S. Roopakala & C. R. Wilma Delphine Silvia & K. M. Prasanna Kumar


Abstract The aim of the study was to assess the plasma fibrinogen levels in type 2 diabetes mellitus patients and to compare with that of healthy controls. To correlate plasma fibrinogen levels with blood pressure (BP), BMI (Body Mass Index) and HbA1c (Glycated hemoglobin) among diabetics and healthy controls. Blood samples were collected for Plasma fibrinogen levels and HbA1c from 30 diagnosed type 2 diabetes mellitus patients. Their BP and BMI were recorded. 30 age and sex matched healthy subjects were included as a control group. There was a rise in plasma fibrinogen levels in diabetics with BMI < 25 kg/m² as compared to controls of the same BMI group (P00.097). Also, there was a trend of raised plasma fibrinogen levels among male diabetics as compared to male control subjects (P00.079). However, there appears no such difference among female individuals. However, there was no significant correlation between fibrinogen levels, blood pressure and HbA1c both among controls and diabetic cases (P >0.05). Though there was a slight trend of increasing levels of plasma fibrinogen among diabetics as compared to controls. However, a significant positive correlation between BMI, male gender and increasing age with plasma fibrinogen was found.

Keywords Fibrinogen · Diabetes mellitus · Glycated hemoglobin · Endothelium · Body Mass Index

Introduction

Diabetes mellitus is associated with an increased risk of cardiovascular disease, even in the presence of intensive glycemic control. Both diabetes mellitus and insulin resistance cause a combination of endothelial dysfunctions, which may diminish the anti-atherogenic role of the vascular endothelium. Hence, in these patients endothelial dysfunction may be a critical early target for preventing atherosclerosis and other cardiovascular complications [1].

The accelerated macrovascular disease in type 2 diabetes mellitus is due partly to the increased incidence of cardiovascular risk factors, such as hypertension, obesity and dyslipidemia [2]. Vascular endothelium plays a major role in maintaining cardiovascular homeostasis. Diabetes disrupts endothelial integrity through an increased oxidative stress. Endothelial dysfunction may precede the development of overt DM, and a prolonged and repeated exposure to postprandial hyperglycemia may play an important role in the development of atherosclerosis, even in those who have normal fasting plasma glucose levels [3]. The pathogenesis of cardiovascular disease (CVD) in diabetes is multifactorial and can be affected by metabolic and other factors. A hypothesis for the initial lesion of atherosclerosis is endothelial dysfunction, defined pragmatically as changes in the concentration of the chemical messengers produced by the endothelial cell. The way endothelial function is altered in diabetic patients is not yet fully understood, but the loss of normal endothelial function could be involved in the pathogenesis of diabetic angiopathy leading to diabetic microangiopathy and macroangiopathy. However, this excess risk is not completely explained by an increased prevalence of...
the major conventional cardiovascular risk factors like smoking, hypertension and hypercholesterolemia. It has been suspected that fibrinogen is involved in producing the excess rate of cardiovascular disease in patients with non-insulin-dependent diabetes mellitus. Increased plasma fibrinogen concentration was associated with diabetic microvascular disease, in particular with nephropathy which is an important risk factor for cardiovascular disease. This may also help to explain the etiologic link between nephropathy and CVD [4]. Because microalbuminuria has been recognized as a powerful predictor of cardiovascular-related illness and death, fibrinogen level may be considered a potential additional risk factor in patients with diabetes.

Plasma fibrinogen is an important marker of inflammation and is also a potent prothrombotic factor responding to endothelial injury. Over the past few years, attention has been focused on fibrinogen, suggesting its role in the pathogenesis of cardiovascular risks in diabetic patients elevated. Some studies have shown that thrombotic (smoking, low fruit/vegetable intake, fibrinogen and homocysteine) as well as atherosclerotic (hypertension, high fat diet, dyslipidemia) risk factors were important in premature coronary heart disease (CHD) [5]. Fibrinogen may promote, together with other haemostatic factors, atherosclerotic changes and thrombosis through effects shown in vitro on platelet aggregation, blood viscosity and foam cell formation. Nevertheless, not many studies have been undertaken to establish a clear relationship between plasma fibrinogen levels and type 2 diabetes mellitus. The high prevalence of classic cardiac risk factors in patients with non-insulin-dependent diabetes mellitus does not explain the increased cardiovascular-related morbidity and mortality in these patients. Fibrinogen may have a role in this excess risk. This study has opened a whole plethora of new questions related to plasma fibrinogen in association to type 2 diabetes mellitus. Not many studies have been undertaken in the past on this topic, and most of those which were done, failed to arrive at a substantial conclusion. This leaves the area of study even more intriguing and fascinating than ever.

Materials & methods

This is an observational, cross sectional study. The study was carried out at the Department of Endocrinology, M S Ramaiyah Medical and Teaching Hospital. The total number of subjects included were 60 and divided into 2 groups containing 30 subjects in each group. Group 1 consisting of type 2 diabetic patients as per WHO criteria and with >5 years of duration of diabetes, between ages 40 and 60 years. Group 2 consisting of age and sex matched healthy controls. History suggestive of any acute/chronic illness/any infections, trauma or malignancy, history of any anti-inflammatory drug intake, history of any liver disease and diabetes mellitus associated with complications were excluded from this study. Informed consent was taken from all patients. Ethical clearance was obtained from the institutional ethics committee.

After taking aseptic precautions, 5 ml blood was drawn into commercially available citrated vacutainers (9 parts of blood: 1 part of sodium citrate) using disposable needles. This blood sample was divided into 2 parts. One part was used for the assessment of HbA1c levels. The other part was used for the assessment of plasma fibrinogen levels.

The blood sample that was used for the assessment of plasma fibrinogen was centrifuged for 20 min at 3,600 rpm soon after the collection of blood sample from the patient. The clear plasma obtained after centrifugation was used for the fibrinogen estimation.

The Dade Behring Fibrinogen Determination kit (Dade Behring Marburg GmbH Marburg, Germany) was used to assess the plasma fibrinogen levels. The thrombin clotting time of dilute plasma is inversely proportional to the fibrinogen concentration of plasma. Using this principle, a simple quantitative assay for fibrinogen was developed by measuring the clotting time of dilute plasma when excess thrombin is added. The clotting time obtained was then compared with that of a standardized fibrinogen preparation.

The glycated hemoglobin (HbA1c) was measured by using high performance liquid chromatography (D10 Biorad kit, USA).

Height of study subjects was measured using a standardized measuring scale and weight was measured using a standardized weighing machine. Body mass index (BMI) was measured using the formula: BMI (kg/m²) = Weight (kg) / Height² (m²)

Statistical analysis

Statistical analysis was done by using Statistical software SPSS 15.0. Mean ± SD was calculated and Student t test (two tailed, independent) was used to find the significance of study parameters on continuous scale between two groups (inter group analysis). Pearson’s correlation age and BMI between study parameters have been tested by student t test. A value of P ≤0.05 was taken to indicate statistical significance.

Results

There was a rise in plasma fibrinogen levels in cases (292.39±55.17) compared to controls (277.70±55.02)
but not statistically significant (P>0.05) (Table 1). There was a raise in plasma fibrinogen levels in type 2 diabetics with BMI <25 kg/m² as compared to controls of the same BMI group (P<0.001) (Table 2). Also, there was a trend of raised plasma fibrinogen levels among male diabetics as compared to male control subjects (P=0.079). However, there appears no such difference among female individuals. However, there was no significant correlation between fibrinogen levels, blood pressure and HbA1c both among controls and diabetic cases (P>0.05) (Tables 3 and 4).

Discussion

There is a strongly significant rise in systolic blood pressure (SBP) and a rise in diastolic blood pressure (DBP) among diabetic patients as compared to healthy controls. This is consistent with the fact that diabetes mellitus causes various functional changes in the endothelium leading to anatomical changes of the vessel wall, ultimately leading to increased resistance to blood flow manifesting as hypertension. We found a strongly significant rise in SBP among diabetic patients with BMI<25 kg/m² as compared to normal controls. We found a strongly significant rise in glycated hemoglobin levels among diabetic cases as compared to healthy controls. We also found a strongly significant rise in HbA1c levels in diabetics as compared to controls among individuals with BMI<25 kg/m² and overweight individuals. There is a moderately significant increase in HbA1c levels in obese diabetics as compared to obese normal controls. This would emphasize that a low BMI value may not always be associated with improved glycemic control. HbA1c levels were raised in all diabetics as compared to healthy controls irrespective of sex.

It has been shown that the endothelial functions improved sequentially with control of diabetes from fair to good to excellent glycemic control [6]. Also hyperglycemia affects multiple mechanisms that deal with oxidation, thrombosis and inflammation. Hence, we expected a trend of increased fibrinogen levels in diabetics. However, no significant difference in the plasma fibrinogen levels was found between the diabetic cases and the normal control subjects. In this study, brachial artery flow mediated vasodilatation (FMD) was studied in patients with poorly controlled and fairly controlled glycaemia to see the effect of glycemic control on endothelial functions [6]. Thus, it might be possible that in our study, diabetic patients with controlled HbA1c levels were under ‘short-term’ glycemic control. This could have caused a lack to significant positive correlation between fibrinogen and HbA1c levels.

**Table 1**: Comparison of study parameters between controls and cases

<table>
<thead>
<tr>
<th>Study parameters</th>
<th>Controls</th>
<th>Cases</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height (meter)</td>
<td>1.53±0.07</td>
<td>1.56±0.08</td>
<td>0.346</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>60.43±11.15</td>
<td>63.19±10.69</td>
<td>0.401</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.62±4.05</td>
<td>26.08±3.88</td>
<td>0.700</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>118.50±11.88</td>
<td>132.22±12.46</td>
<td>0.001**</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>75.63±4.63</td>
<td>79.22±7.33</td>
<td>0.077</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.17±0.55</td>
<td>8.12±1.73</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Fibrinogen (mg/dl)</td>
<td>277.70±55.02</td>
<td>292.39±55.17</td>
<td>0.379</td>
</tr>
</tbody>
</table>

**Statistically significant**

**Table 2**: Comparison of study parameters according to BMI

<table>
<thead>
<tr>
<th>Study parameters</th>
<th>BMI(kg/m²)</th>
<th>Controls</th>
<th>Cases</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>&lt;25</td>
<td>116.44±10.04</td>
<td>134.13±14.99</td>
<td>0.005**</td>
</tr>
<tr>
<td></td>
<td>25–30</td>
<td>125.00±18.51</td>
<td>131.87±10.86</td>
<td>0.345</td>
</tr>
<tr>
<td></td>
<td>&gt;30</td>
<td>116.00±5.29</td>
<td>128.33±9.83</td>
<td>0.087</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>0.480</td>
<td>0.635</td>
<td>–</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>&lt;25</td>
<td>73.55±4.67</td>
<td>78.27±9.32</td>
<td>0.174</td>
</tr>
<tr>
<td></td>
<td>25–30</td>
<td>77.50±4.12</td>
<td>79.20±6.18</td>
<td>0.613</td>
</tr>
<tr>
<td></td>
<td>&gt;30</td>
<td>79.33±1.15</td>
<td>81.67±4.06</td>
<td>0.378</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>0.106</td>
<td>0.644</td>
<td>–</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>&lt;25</td>
<td>5.08±0.57</td>
<td>8.77±2.33</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td></td>
<td>25–30</td>
<td>5.18±0.17</td>
<td>7.61±0.73</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td></td>
<td>&gt;30</td>
<td>5.43±0.91</td>
<td>7.78±1.42</td>
<td>0.037*</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>0.676</td>
<td>0.163</td>
<td>–</td>
</tr>
<tr>
<td>Fibrinogen (mg/dl)</td>
<td>&lt;25</td>
<td>266.27±55.75</td>
<td>305.34±57.98</td>
<td>0.097</td>
</tr>
<tr>
<td></td>
<td>25–30</td>
<td>266.60±32.82</td>
<td>284.93±60.25</td>
<td>0.571</td>
</tr>
<tr>
<td></td>
<td>&gt;30</td>
<td>326.80±64.81</td>
<td>278.72±57.80</td>
<td>0.263</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>0.242</td>
<td>0.493</td>
<td>–</td>
</tr>
</tbody>
</table>

*Statistically significant (P<0.05)

**Statistically highly significant (P<0.001)
We found no notable difference in plasma fibrinogen levels among different age groups in both diabetic patients and normal controls. This tells us that age may not play a role in determining levels of proinflammatory markers in diabetes. However, many previous studies have opined on the contrary. For example, proinflammatory markers associated with an increased risk of adverse cardiovascular outcomes have been shown to be present already in obese adolescent youth with insulin resistance [7]. Thus, this would require further investigations to arrive at a firm conclusion.

We found a trend of raised plasma fibrinogen levels in diabetics with BMI<25 kg/m² as compared to controls of the same BMI group. However, there appears no difference in plasma fibrinogen levels between diabetics and controls among both overweight and obese individuals. Hence, this is another area into which we need to research in future.

There is a trend of raised plasma fibrinogen levels among male diabetics as compared to male control subjects. However, there appears no such difference among female individuals. This suggests that there is a subtle correlation between sex and fibrinogen levels. Not many studies have been undertaken in this regard, especially in type 2 diabetic patients. This is another area in which research could be undertaken. A study could be performed to look into the influence of female sex hormones on plasma fibrinogen levels. We found no significant correlation between fibrinogen levels, blood pressure and HbA1c. This was also noted by other investigators [8]. A study reported the effect of insulin therapy on coagulation and fibrinolysis in critical care patients. There was no effect of insulin therapy on any of the fibrinolytic, coagulation, or inflammatory parameters tested [9]. Thus, the relationship between glycemic control and plasma fibrinogen has been rather controversial. Further investigation into this regard may yield us valuable information.

In conclusion, diabetes mellitus is associated with chronic inflammation leading to endothelial dysfunction eventually leading to peripheral vasculopathies, which reflects as raised plasma levels of inflammatory and prothrombotic markers. However, a clear relationship between plasma fibrinogen levels, which is both a proinflammatory and a prothrombotic marker, and glycemic control could not be established in this study. There is a positive correlation between increasing age and raised plasma fibrinogen levels in diabetic patients. Also, it has been found that male diabetics are prone to have higher plasma levels of fibrinogen. We found a rise in fibrinogen levels among diabetics with BMI<25 kg/m². However, no correlation was established between glycemic control and plasma fibrinogen levels in this study. Since this has been a topic of controversy over the past few years, it may require further research studies to arrive at a final conclusion. Research directed towards studying the relationship between fibrinogen and the influence of female sex hormones in type 2 diabetics will be of valuable help.

Table 3  Comparison of study parameters according to gender

<table>
<thead>
<tr>
<th>Study parameters</th>
<th>Gender</th>
<th>Controls</th>
<th>Cases</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>Male</td>
<td>118.57±7.18</td>
<td>134.00±9.13</td>
<td>0.001**</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>118.44±15.03</td>
<td>130.44±15.15</td>
<td>0.063</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>0.984</td>
<td>0.400</td>
<td>–</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>Male</td>
<td>75.43±3.41</td>
<td>78.67±6.79</td>
<td>0.245</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>75.78±5.61</td>
<td>79.78±7.99</td>
<td>0.1936</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>0.887</td>
<td>0.656</td>
<td>–</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>Male</td>
<td>5.40±0.72</td>
<td>8.01±1.11</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>5.00±0.32</td>
<td>8.23±2.21</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>0.157</td>
<td>0.713</td>
<td>–</td>
</tr>
<tr>
<td>Fibrinogen (mg/dl)</td>
<td>Male</td>
<td>246.40±45.09</td>
<td>293.07±60.67</td>
<td>0.079</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>302.04±57.25</td>
<td>291.73±50.84</td>
<td>0.624</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>0.040*</td>
<td>0.943</td>
<td>–</td>
</tr>
</tbody>
</table>

*Statistically significant (P≤0.05)

**Statistically highly significant (P≤0.001)

Table 4  Pearson’s correlation of fibrinogen with study parameters

<table>
<thead>
<tr>
<th>Correlation between</th>
<th>SBP</th>
<th>DBP</th>
<th>HbA1c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen</td>
<td>Control</td>
<td>−0.118</td>
<td>0.133</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>(0.663)</td>
<td>(0.62)</td>
</tr>
<tr>
<td>Cases</td>
<td>r value</td>
<td>−0.078</td>
<td>−0.104</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>(0.65)</td>
<td>(0.547)</td>
</tr>
</tbody>
</table>

Conflict of interest  We declare that we have no conflict of interest.

References

Free radicals are molecules, which lack an electron in their chemical structure, which they constantly try to replace. The free radicals try to get electrons from the body, which causes “oxidative stress,” which in turn results in damage to the body’s cells and tissues. Antioxidants provide the extra electrons to the free radicals and prevent them from causing damage to the body’s cells and tissues. Since world war II the free radicals biochemistry came into vogue. Free radicals have been implicated in the etiology of several human diseases as well as ageing [1, 2].

Halliwell & Gutteridge (1989) [3] state that an antioxidant is “any substance that, when present at low concentrations compared with that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate”. This definition includes reactions of a non-enzymatic as well as an enzymatic nature. There are many protective antioxidant mechanisms like- superoxide dismutase, catalase, glutathione, glutathione peroxidase, reductase and non-enzymatic antioxidants like carotenoids, flavonoids, trace elements like zinc, selenium, probucol, vitamins like vitamin E and vitamin C (ascorbic acid), which are available in nature. Fruits, vegetables, miner- als, tea and nuts are good source of antioxidants.

Free radicals by lipid peroxidation adversely affect lipids, proteins and DNA. Free radical can damage the proteins, which results in the loss of enzyme activity. Damage caused to DNA, can result in mutagenesis and carcinogenesis [4]. Antioxidants like phenols, flavonoids, isoflavones, isothiocyanates, diterpenes, methylxanthines, dithiols, and coumarins appear to be important in cancer prevention [5].

The epidemiological studies showed that higher intake of foods with antioxidant can prevent and control certain diseases in humans. Many natural products and Indian spices and ayurvedic products are known to possess antioxidant activity. Many epidemiological studies have shown association between consumption of vitamins and anti oxidants and reduction in cardiovascular diseases and its morbidity and mortality. These are mostly observational studies.

An article by Shital S Panchal on Anti-diabetic activity of oryzanol and its relationship with the anti-oxidant property, in this issue [6] showed that oryzanol (OZ), a commercially-important bioactive phytochemical isolated from crude rice bran oil (cRBO), possesses the potential to effectively ameliorate the oxidative stress in diabetic rats. Translating the knowledge and experiments from bench to bedside is not an easy task.

There are many publications on the role of antioxidants and their efficacy in preventing or curing metabolic disorders like diabetes mellitus, inflammatory diseases like periodontitis, aging diseases like ischaemic heart diseases, cancer and degenerative diseases like Alzheimers disease, Parkinsonism. There are very few RCT’s in human beings to prove the efficacy of antioxidants in prevention, control or cure of chronic diseases. One of the clinical studies with probucol an antioxidant, in 317 patients showed probucol, is effective in reducing the rate of restenosis after balloon coronary angioplasty [7].

A literature search of pubmed by Saeid Golbidi, et al. [8] for effect of antioxidants like vitamin E, vitamin C, CoQ10, alpha lipolic acid, L-carnitine, ruboxistaurin or LY 333531 in diabetes mellitus, revealed that routine vitamin or mineral supplementation is not generally recommended in human diabetes.

Tocopherol and Coenzyme Q10 have been tried in prevention and treatment of Parkinson’s disease with limited success. A review of antioxidants in Parkinson’s disease [9] concluded that antioxidants have limited role in the prevention and treatment. They are also of the opinion that CoQ10 has some minor benefits in Parkinson disease.

Though there is plenty of literature on the role of antioxidants in etiopathogenesis of disease in human beings, is the evidence enough to use antioxidants in clinical practice to prevent, control and “cure” diseases and reduce the mortality and morbidity? Another issue is dose of antioxidants and use of combination therapy. Translating the dose of...
antioxidants to human trials is not an easy issue. Each study has used different doses of antioxidant. Is there a dose-effect relationship between the dose of antioxidant for prevent, control and cure of disease? Does one size fit all? Is the combination of two or more antioxidants better than the summed benefits of each antioxidant. There is no simple answer for these questions in evidence based medicine. We need to wait for larger and longer studies in human beings on the effect of antioxidants in preventing disease, promoting health and reducing morbidity and mortality of acute and chronic illnesses.

Future research like gene therapy to enhance production of antioxidants in the human body, genetically engineered plant products with higher concentrations of antioxidants, synthetic antioxidant enzymes or non enzymatic chemical molecules, newer peptides with antioxidant properties developed by recombinant DNA technology and computational chemistry and functional foods enriched with antioxidants will replace the present anti-oxidant remedies.

References

Hypoglycemic and hypolipidemic activity of Hibiscus rosa sinensis Linn on streptozotocin–induced diabetic rats

Anusha Bhaskar & V. G. Vidhya


Abstract To investigate the hypoglycemic activity of an aqueous extract of the flower of Hibiscus rosa sinensis on blood glucose in normal and streptozotocin (STZ) -induced diabetic rats, serum triglyceride and cholesterol levels and the effect of the flower extract on insulin secretion. Effect of H. rosa sinensis flower extract on blood glucose was studied with fed, fasted and glucose-loaded diabetic and nondiabetic rats. Glycosylated hemoglobin, serum insulin levels and lipid profile were also determined. Student’s t test was used for statistical analysis. In normal rats, the aqueous extract of the flower of H. rosa sinensis (250 and 500 mg/kg body weight) significantly (P<0.001) reduced the blood glucose levels after an oral glucose load from 127.9±5.6 to 80.6±3.9 mg/dl 2 h after oral administration of the flower extract. It also significantly lowered the blood glucose in STZ diabetic rats from 241.0±6.6 to 90.8±5.7 mg/dl after 21 days of oral administration of the extract (P<0.001). Serum insulin levels were not stimulated in the animals treated with the extract. Glycosylated hemoglobin and serum lipid profiles were significantly lowered by the administration of the extract. From the studies, it can be concluded that the aqueous extract of the flowers of H. rosa sinensis at a dosage of 500 mg/kg/day exhibits significant hypoglycemic and hypolipidemic activities. A marked reduction in glycosylated hemoglobin was also observed while insulin levels did not show any significant change.

Keywords Hibiscus rosa sinensis · Streptozotocin-induced diabetes mellitus · Hypoglycemic activity · Hypolipidemic · Blood glucose

Introduction

Many indigenous Indian plants have been shown to possess antihyperglycemic activity and proven to be useful in the management of diabetes mellitus. One such plant is Hibiscus rosa sinensis. It belongs to the family Malvaceae, it is a glabrous shrub widely cultivated in the tropics as an ornamental plant and has several forms and varying colours of flowers [1]. It is also used in traditional medicine to induce abortion, ease menstrual cramps, assist in childbirth and relieve headache, fever and inflammation [2]. Sachdewa and Khemani [3] reported the hypoglycemic activity of Hibiscus rosa sinensis flower extract. In addition to the hypoglycemic role we have studied the effect of H. rosa sinensis on the insulin levels in diabetic rats.

Materials and methods

Male Wistar rats (weighing 150–200 g) were purchased from Bharathidasan University, Tiruchirappalli and were maintained under standard environmental conditions (12:12 h light dark cycles) and fed with a standard diet (Hindustan Lever, India) and water ad libitum. All the studies were conducted in accordance with the National Institute of Health guide [4]. The work was carried out in CPCSEA approved (743/03/abc/CPCSEA dt. 3.3.03) Animal House of PRIST University, Thanjavur.

Hibiscus rosa sinensis flowers were collected from Trichy, Tamil Nadu. The plant was authenticated and molecular
taxonomy of the plant was done by sequencing the 18S rDNA of the plant and the sequence submitted in the Genbank (Acc. No: FJ665614).

Preparation of plant extract

The flowers of *H. rosa sinensis* was shade dried, powdered and stored in an air-tight container until further use. The powder was suspended in 500 ml of sterile distilled water and extracted. The solvent was evaporated in vacuo at 50 °C giving an aqueous extract (yield: 21 % w/w). Preliminary phytochemical screening was done to show the presence or absence of secondary metabolites according to methods described by Harbourne [5].

Acute toxicity studies

Healthy adult Wistar albino rats starved overnight were divided into four groups (NO6) and were orally fed with the alcoholic extract in increasing dose levels of 100, 500, 1000 and 3000 mg/kg body weight [6]. The rats were continuously monitored for 2 h for any behavioral, neurological and autonomic profiles and after 24 and 72 h for any lethality [7].

Assessment of hypoglycemic activity in normal healthy rats

The animals that were fasted overnight were divided into four groups of 6 animals each. Control rats (group I) were given vehicle (distilled water) only, while groups II and III received the *H. rosa sinensis* flower aqueous extract suspended in distilled water orally at doses 250 and 500 mg/kg, respectively [8]. The fourth group received 250 mg/kg of tolbutamide, used as a reference standard drug. Blood glucose levels were estimated before and after 1, 2, 4 and 6 h of flower extract administration.

Assessment of anti-hyperglycemic activity in normal rats: oral glucose tolerance test (OGTT) [9]

After overnight fasting, the rats were given the reference drug and test extracts orally and 30 min later, glucose (10 g/kg) was administered orally. Blood samples were collected before and at 30, 60 and 120 min after glucose administration.

Assessment of plant extract on STZ diabetic rats

Rats were made diabetic by single intraperitoneal administration of streptozotocin (55 mg/kg) dissolved in 0.1 M citrate buffer, pH 4.5. Forty eight hours later, blood samples were collected and glucose levels were determined to confirm the development of diabetes. Only those animals which showed hyperglycemia (blood glucose levels >240 mg/dl) were used in the experiment. The diabetic rats were divided into five groups of six animals each. Group I received vehicle alone and served as control dissolved in 0.1 M citrate buffer. Groups II and III received the *H rosa sinensis* flower aqueous extract (250 and 500 mg/kg/day for 6 weeks suspended in vehicle. Group IV received tolbutamide (250 mg/kg). Blood samples were drawn at 1, 2, 4 and 6 h after extract administration and then at weekly intervals till the end of the study.

The effect of administration of the aqueous extract of *H. rosa sinensis* on diabetic rats were estimated on the 21st day after the animals were sacrificed by decapitation. Blood glucose was estimated by glucose oxidase method [10]. Serum insulin levels (Radioimmunoassay kit, Diasorin, Italy), glycosylated hemoglobin (BioSystems, Costa Brava, Spain), and serum lipid profiles—serum cholesterol (Beacon Diagnostics, Kabilpore, India), triglycerides (Bio Systems, Costa Brava, Spain) and HDL cholesterol (Beacon Diagnostics, Kabilpore, India) were

<table>
<thead>
<tr>
<th>Treatment mg/kg body wt</th>
<th>Blood glucose mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>Fasted normal</td>
<td>63.8±3.6</td>
</tr>
<tr>
<td>Normal control</td>
<td>64.1±4.4</td>
</tr>
<tr>
<td>Hibiscus rosa sinensis 100 mg</td>
<td>64.0±2.5</td>
</tr>
<tr>
<td>Hibiscus rosa sinensis 250 mg</td>
<td>64.2±3.2</td>
</tr>
<tr>
<td>Tolbutamide 250 mg</td>
<td></td>
</tr>
</tbody>
</table>

*Values are statistically significant at P < 0.01; **Values are statistically significant at P < 0.0001. Experimental groups were statistically compared with the corresponding values at the controls.
Table 3 Effect of H. rosa sinensis aqueous extract on oral glucose (10 g/kg) tolerance test in normal control rats (mean ± S.D.)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Blood glucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fasting</td>
</tr>
<tr>
<td>Oral glucose tolerance test</td>
<td></td>
</tr>
<tr>
<td>Control + 10 g/kg glucose</td>
<td>68.4±1.9</td>
</tr>
<tr>
<td>H. rosa sinensis</td>
<td>67.8±2.4</td>
</tr>
<tr>
<td>500 + 10 g/kg glucose</td>
<td>67.0±4.9</td>
</tr>
<tr>
<td>Tolbutamide 250 + 10 g/kg glucose</td>
<td>6.8±4.2</td>
</tr>
</tbody>
</table>

Values are statistically significant at P<0.01; **Values are statistically significant at P<0.001. Experimental groups were statistically compared with the corresponding values of the controls.

Results

Phytochemical analysis of the extract showed the presence of alkaloids, flavonoids, steroids, glycosides and polyphenols as shown in Table 1.

Acute toxicity tests revealed that the extract is non-toxic in nature as there were no lethality or toxic reactions found at any dose selected for the study. The aqueous extract showed a significant reduction in blood glucose levels from 30 min onwards. The effects of the treatment with H. rosa sinensis aqueous extract and tolbutamide on blood glucose concentration in normal fasted and diabetic rats after acute treatment are shown in Table 2. Control rats treated with 500 mg/kg showed a significant fall in blood glucose by 25.2 % after 6 h of oral administration. A 23.9 % change was observed with tolbutamide.

The hypoglycemic effect of the H. rosa sinensis flower aqueous extract (500 mg/kg) was comparable to that of tolbutamide treated rats (37 %) (Table 3).

Diabetic rats treated with 250 and 500 mg/kg of the H. rosa sinensis aqueous flower extract showed a significant reduction in blood glucose levels of 12 and 18 % respectively, after 6 h of treatment (Table 4).

Daily treatment for 3 weeks with H. rosa sinensis aqueous flower extract (250 and 500 mg/kg) brought about a significant fall in the blood glucose levels by 54 and 62 % respectively. At the same time tolbutamide caused a significant reduction in blood glucose by 59 % (Table 5). The serum insulin levels were not stimulated and the results are presented in Table 6. Significant difference was observed in glycosylated hemoglobin (Table 6) and serum lipid profiles (Table 7).

Discussion

The ever increasing number of diabetic patients has motivated the scientists to find new methods of treatment to control diabetes. Our study suggests that the crude extract of the flower of H. rosa sinensis displayed significant hypoglycemic effect, the main mechanism by which it does is probably by stimulating peripheral glucose consumption, since the extract did not have an effect on insulin stimulatory function. Hence, the hypoglycemic effect may be probably brought about by an extra pancreatic mechanism.

A continuous treatment for 21 days with the aqueous extract caused a significant decrease in blood glucose levels estimated in all the four groups and the values of the treated groups were compared with controls and diabetic controls.

All experimental data were expressed as mean ± S.D. The difference between test and controls were evaluated by Student’s t-test [11].
of diabetic rats but no such effect was observed in the control rats. Therefore, a continuous administration of the extract may not lead to hypoglycemia as is observed with an overdose of insulin or sulfonylurea drug [12].

The dose of 500 mg/kg of the aqueous extract not only lowered the total cholesterol and triglyceride levels but also enhanced the levels of HDL suggesting cardioprotective role of the extract. Hyperlipidemia is implicated in the development of atherosclerosis [13]. The underlying mechanism of H. rosa sinensis in lipid lowering may be by inhibition of lipid absorption due to the presence of saponins and polyphenols. Studies show that the oral administration of saponins from some medicinal plants significantly reduce triglycerides and cholesterol levels in rats [14]. HDL functions in transporting cholesterol away from the peripheral tissue to the liver, thus preventing the progression of atherosclerosis. We observed a significant elevation in HDL level which points to the cardioprotective function of H. rosa sinensis. Significant lowering of total cholesterol and a rise in HDL cholesterol is a very desirable biochemical state for the prevention of atherosclerosis and ischaemic conditions [15].

This experimental study reveals that streptozotocin-treated animals who received H. rosa sinensis extracts showed lower blood glucose level as compared to the diabetic group. This may be due to the possibility that few of the beta cells are still surviving and stimulated by the extract component(s), releasing insulin. The aqueous extract of H. rosa sinensis flower extract contains active secondary metabolites which include alkaloids, flavonoids, saponins, polyphenols and which are considered to be bioactive for the management of diabetes. It is known that certain alkaloids exhibit hypoglycemic activity and are also known for their ability of beta cell regeneration of pancreas [16]. Polyphenols have also shown to decrease blood glucose; the antidiabetic activity of H. rosa sinensis extract may be due to the presence of one or more of the antihyperglycemic principles and their synergistic effects.

In conclusion our results showed that Hibiscus rosa sinensis extract markedly reduced hyperglycemia in STZ-induced diabetic rats, decreased plasma triglycerides and total cholesterol and increased HDL-cholesterol. These findings suggest that Hibiscus rosa sinensis extract is useful in the control of diabetes mellitus.

### Table 5

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Blood glucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
</tr>
<tr>
<td>Control</td>
<td>67.5±2.0</td>
</tr>
<tr>
<td>STZ treated</td>
<td>67.8±1.8</td>
</tr>
<tr>
<td>Hibiscus rosa sinensis (250 mg/kg/body weight)</td>
<td>67.5±2.0</td>
</tr>
<tr>
<td>Hibiscus rosa sinensis (500 mg/kg/body weight)</td>
<td>66.9±1.8</td>
</tr>
<tr>
<td>Tolbutamide (250 mg/kg/body weight)</td>
<td>65.7±1.6</td>
</tr>
</tbody>
</table>

"Values are statistically significant at P<0.001. STZ treated was compared with the normal control and experimental groups were compared with the corresponding values at 48 h.

### Table 6

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Insulin (μU/ml)</th>
<th>Glycosylated hemoglobin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (non diabetic)</td>
<td>124.8±12.8</td>
<td>3.5±0.6</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>96.4±9.4</td>
<td>7.2±0.6</td>
</tr>
<tr>
<td>H. rosa sinensis 250 mg/kg bw</td>
<td>101.5±5.6</td>
<td>4.8±0.5 &quot;a,b&quot;</td>
</tr>
<tr>
<td>H. rosa sinensis 500 mg/kg bw</td>
<td>100.1±8.2</td>
<td>4.2±0.2 &quot;a,b&quot;</td>
</tr>
</tbody>
</table>

"a" Represents statistical significance vs. control (P<0.05)

"b" Represents statistical significance vs. diabetic control (P<0.05)

### Table 7

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Triglyceride mg/dl</th>
<th>Total cholesterol mg/dl</th>
<th>HDL cholesterol mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (non diabetic)</td>
<td>93.2±1.5</td>
<td>59.1±2.4</td>
<td>25.02±1.33</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>185±12.4</td>
<td>124.5±14.1</td>
<td>12.14±2.20</td>
</tr>
<tr>
<td>H. rosa sinensis 250 mg/kg bw</td>
<td>104.8±15.2 &quot;a,b&quot;</td>
<td>70.5±1.3 &quot;a,b&quot;</td>
<td>22.04±2.15 &quot;a,b&quot;</td>
</tr>
<tr>
<td>H. rosa sinensis 500 mg/kg bw</td>
<td>82.4±7.0 &quot;a,b&quot;</td>
<td>61.5±4.6 &quot;a,b&quot;</td>
<td>23.50±2.36 &quot;a,b&quot;</td>
</tr>
</tbody>
</table>

"a" Represents statistical significance vs. control (P<0.05)

"b" Represents statistical significance vs. diabetic control (P<0.05)
Conflict of interest  All authors declare that they have no conflicts of interest

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4. National Institute of Health Guide for the Care and Use of Laboratory animals. DHEW Publication (NIH), revised, Office of Science and Health Reports, DRR/NIH, Bethesda, USA; 1985.
Abstract The postprandial metabolic response to whole foods is influenced by the structural, functional, nutritional properties, processing conditions as well as the effect of co-nutrients. In the present study, glycemic and insulin response to products made from wheat flour was determined by feeding identical portions of test foods and standard. Equi-quantity based comparison of foods is expressed by ‘Relative Glycemic Impact (RGI)’ wherein the amount of bread (reference food) that produces similar response as that of the given amount of test food is represented as Glycemic Bread Equivalent (GBE). Five clinically healthy adult volunteers were fed 50 g of food products such as Chapatti (Indian flatbread), Thepla (wheat flour, Bengal gram flour and fat), Marie biscuit (Wheat flour and fat) and Bread on different days. Blood samples were drawn at fasting (0 min) and 30, 60, 90 and 120 min after consuming food and the corresponding incremental area under the curve (IAUC) was calculated. The GBE/50 g of Marie biscuit (44.3 g) and chapatti (43.8 g) was much higher than that of Thepla (17 g) (P<0.05). The overall insulinemic effect of Thepla (3.5 g) was also found to be much lower than that of equal amount Marie biscuit (58 g) (P<0.01) and chapatti (39 g) (P<0.05). Thepla induced the lowest hyperglycemic and hyperinsulinemic effect indicating that it is a better choice for the overweight, insulin resistant as well as diabetic group. RGI (GBE/g of food) values reflect the importance of nutrient composition of end-products in affecting overall glycemic response to whole foods.

Keywords  Wheat products - Relative Glycemic Impact - Glycemic Bread Equivalent - Insulinemic effect

Introduction
The postprandial glycemic response to foods is a function of the quality and quantity of carbohydrates present. Glycemic Index expresses the qualitative response to foods by comparing 50 g available carbohydrate portions of test foods and standard [1]. Factors such as type of sugar, starch composition, presence of moisture, co-ingredients like protein, fat, fiber, method of cooking or processing and quantity consumed may influence glycemic responses to a great extent besides the available carbohydrate content.

The glycemic effect of given quantity of selected processed food as a whole as consumed by the individual has been assessed in the present study. The equi-quantity based comparison of whole foods is done by using the concept of ‘Relative Glycemic Impact (RGI)’ [2]. The RGI is defined as the blood glucose response elicited by a specific quantity of test food compared with that of same quantity of a standard such as glucose or white bread to give Glycemic Glucose Equivalent (GGE) or ‘Glycemic Bread Equivalent (GBE)’ respectively [3]. The terminology is used to represent acute effects of single intake (food weight) or serving [2] expressed as GBE/ amount of food. Therefore, GBE/50 g is the weight of bread that would induce a glycemic response equal to that induced by 50 g of the test food [3]. These values are expressed in gram units and can be placed in the food exchange list alongside other nutrients. The
predicted glycemic effect of common serving sizes of the test foods can also be calculated to aid practical nutrition counseling.

The postprandial glycemic effect depends directly on insulin response to the food product. Hence, the Insulinemic Bread Equivalent (IBE) values were also calculated by comparing 2 hour postprandial insulin response to equal quantities of test foods and standard.

India is the second largest producer of wheat in the world, averaging an annual production of 65,856 TMT and consumption of 65,283 TMT as reported by the USDA Economic and Statistics System and the USDA/NOAA Joint Agricultural weather facility. Wheat kernels are ground to different degrees yielding flour of varying thickness. Whole wheat flour retains more fiber than refined flour. Most Indian breads are prepared using wheat flour kneaded with water rolled into chapatti, phulka, or roti varying in size, thickness and other physical characteristics. The glycemic and insulin response to whole wheat chapatti (without fat) has been studied in order to establish the benefits of this staple food product on postprandial metabolism in normal healthy individuals.

Thepla, a variant of the chapatti, is a typical specialty from State of Gujarat served with curds, pickle or chutney. It forms a satisfying mini meal by itself due to presence of several ingredients such as Bengal gram flour, fenugreek leaves, curds and sometimes even jaggery, which improve its overall nutritional value. The postprandial glycemic and insulin responses to thepla have been examined to understand its differential effect compared to pure whole wheat chapatti.

Marie biscuit is prepared using refined wheat flour (maida) kneaded into dough by adding vanasperi/hydrogenated oil before being baked at high temperatures. Marie biscuit is one of the most popular biscuits, being advised by doctors and nutritionists as a low calorie, light, non-sweet tasting biscuit which is consumed with tea/coffee by most individuals. The crispy and crunchy texture of biscuits is obtained using finely ground wheat flour and vanasperi which renders it low in fiber and also increases the saturated fat content.

In the present study, the Relative Glycemic Impact of wheat and wheat products such as Chapatti, Thepla and Marie biscuit were compared to categorize foods that produce better glycemic control.

Each subject consumed 50 g of each of the three test foods and standard white bread after an overnight fast (>10 h) on different occasions with a gap of at least 5–7 d between each testing. Venous blood samples were drawn in fasting (0 min) state and at 30, 60, 90 and 120 min after consuming food. The subjects were given 10 min to complete the meal. 250 ml of drinking water was given along with the food.

The food samples were analyzed for carbohydrate [4], protein [5], fat [6], fiber (AOAC 18th Edition 2007, 985.29) and moisture content [7]. Venous blood was analyzed for blood glucose using a glucometer (Sugar scan manufactured by HMD Biomedical Inc.) and serum insulin was measured using radio-immunoassay [8].

\[
\text{GI}_{\text{food}} = \frac{\text{RGI} \times \text{AUC blood glucose response to relevant food portion}}{\text{TAUC blood glucose response to equal weight of bread}}
\]

\[
\text{AUC} = \text{Incremental Area Under the Curve}
\]

Therefore, RGI 0 GBE/amount of food, here GBE/50 g of food

The postprandial blood glucose and insulin response areas above the basal were calculated applying trapezoid rule. Results are expressed in Mean ± standard errors (SE). Statistical analysis was performed using one way ANOVA test to test differences in mean total glycemic and insulin AUC responses to the three test foods. Paired sample ‘t’ test was also applied to compare mean differences among IAUC between test foods or test foods and reference. The level of statistical significance was set to 0.05.

Bread Fifty grams or 3 ¼ slices of commercially prepared white wheat bread (Britannia) was served fresh on the day of the testing. Bread is used as standard food for food based comparison [9], as compared to glucose which is used as standard in nutrient-based comparison, e.g. Glycemic Index determination.

Chapatti Thirty-seven grams whole wheat flour (not sieved) was mixed with a pinch of salt and water (30 ml) and gradually kneaded to obtain smooth dough. The dough was allowed to stand for about 30 min. It was then rolled out into chapatti of uniform diameter and thickness and placed on the pan for initial roasting. It was turned over after 15 sec and allowed to cook for another 15 sec after which it was roasted over open medium flame for 10–15 sec till completely done. The cooked chapatti was weighed as 50 g and served fresh for consumption.

Methi Thepla Twenty-five grams whole wheat flour (not sieved) and 7 g of ready Bengal gram flour (besan) were weighed along with 10 g of finely chopped fenugreek leaves
(washed under running tap water and cleaned thoroughly). The dough was kneaded using 7 g curd and little water. A pinch of salt and red chilli powder was added. All the ingredients were mixed to prepare soft non-sticky dough. The dough was allowed to stand for 30 min and then rolled out into Thepla of uniform diameter and placed on the pan for initial roasting. Half of the measured amount of oil (1.5 g) was applied to one side of the thepla and allowed to cook on the pan at medium flame. It was turned over after 15 sec and then rest of the oil was applied to other side and allowed to cook for another 15 sec. The cooked theplas were weighed as 50 g and served fresh for consumption.

Marie Biscuit Fifty grams (9 in number) of commercially prepared biscuit (Britannia Marie Gold) were weighed and fed to the subjects.

Results

The postprandial glycemic response to 50 g of local food products made from wheat flour such as Chapatti, Thepla, and Marie biscuit was compared to that of standard food, white bread.

As seen in Fig. 1, the peak glycemic response was observed in the first 30 min for all the test foods with highest response to chapatti followed by Marie biscuit and least for Thepla. With Thepla and Marie biscuit, the blood glucose levels dropped to fasting at 45 and 90 min respectively. However, the glycemic response curve for chapatti and bread returned to baseline only by 120 min. The GBE/50 g was 44.3 g for Marie biscuit, 43.8 g for chapatti and only 17 g for Thepla. This indicates that Marie biscuit, made with refined flour, does not produce lower relative glycemic effect; but equal to that of chapatti. On the other hand, relative glycemic potency of Thepla is significantly lower than that of Marie biscuit and Chapatti (P < 0.05) and may be a better option for diabetic and obese population.

The 2-hour insulin response was found to be highest for Marie biscuit followed by Chapatti and least for Thepla (Fig. 2). The IBE/50 g for Marie biscuit (58 g) was found to be significantly higher than that of equal amount of Thepla (3.5 g) (P < 0.01). The serum insulin levels for Marie biscuit and Chapatti did not return to baseline even at the end of 120 min. IBE/50 g for chapatti was found to be 39 g which is also higher than that of Thepla but lower compared to Marie biscuit.

Discussion

The test foods as well as standard white bread, are products made from wheat flour undergoing different conditions of cooking/processing with varied nutrient composition.

Figure 1 shows that Chapatti produced elevated blood glucose response which is only slightly less than that of bread [10]. The higher proportion of amylase (25 %) chains in wheat may lead to the slow and sustained release of blood glucose in postprandial period. Despite the prolonged hyperglycemic effect chapatti, prepared from whole wheat flour containing relatively more fiber and protein (Table 1), makes a better choice than biscuits, which is rich in fat (especially saturated fat).

Marie biscuit is prepared using refined wheat flour and contains high amount of starch on wet weight basis (Table 1) and free sugars which are rapidly digested and absorbed causing increased hyperglycemia followed by drop in blood glucose levels within 90 min of consumption (Fig. 1). Nevertheless, Marie biscuit which contains the least amount of fiber and highest fat compared to the other test foods (Table 1) may not be the ideal snack options in obese as well as diabetic population. Only advantage to Marie biscuit is it is rarely consumed in such large quantity at one meal time.

The lower glycemic potency of Thepla may be attributed to its lower starch content on wet weight basis.
Table 1 Proximate composition of the selected wheat products

<table>
<thead>
<tr>
<th>Food product</th>
<th>Moisture (g%)</th>
<th>Total starch</th>
<th>Soluble sugar</th>
<th>Fat (g%)</th>
<th>Protein (g%)</th>
<th>TDF (g%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bread, white</td>
<td>36.8±0.5</td>
<td>52.6</td>
<td>33.6</td>
<td>2.5</td>
<td>8.4±0.7</td>
<td>4.04</td>
</tr>
<tr>
<td>Chapatti</td>
<td>33.3±2.5</td>
<td>47.9</td>
<td>34.1</td>
<td>2.2±0.7</td>
<td>11.1±1.2</td>
<td>5.92</td>
</tr>
<tr>
<td>Thepla</td>
<td>18.6±0.6</td>
<td>32.9</td>
<td>28.2</td>
<td>6.3±0.2</td>
<td>9.2±0.2</td>
<td>7.11</td>
</tr>
<tr>
<td>Marie biscuit</td>
<td>4.5±0.9</td>
<td>46.2</td>
<td>43.2</td>
<td>9.9±0.3</td>
<td>8.5±0.5</td>
<td>1.44</td>
</tr>
</tbody>
</table>

TDF Total Dietary Fiber

(Table 1). The preparation of thepla involves use of Bengal gram flour (25 % of total weight of wheat flour) added to whole wheat flour and small amounts of fresh fenugreek leaves. This increases the protein and fiber content, reducing total starch present in that of 50 g portion of the product (Table 1). Chandalia et al [11] also reported that GI of wheat flour chapatti (86 %) was found to be much higher than that of wheat flour+gram flour (2:1 chapatti (66.4 %). The lower glycemic AUC in response to legume-based foods like Thepla may be due to higher food viscosity [12], high un-absorbable carbohydrates [13] and delayed gastric emptying [14].

Insulin response

The high postprandial insulimemic effect of Marie biscuit and chapatti can be explained by their higher glycemic response. The persistent high insulin levels in postprandial state may promote fat storage and increase risk of developing insulin resistance [15]. The IAUC insulin response to Thepla was found to be significantly lower than that of Marie biscuit (P<0.01). High fiber content and lower starch content in the portion size of Thepla may have led to lowering of insulin levels [16, 17]. Hyperinsulinemia has been associated with increased risk of hypertriglyceridemia and coronary artery disease [18].

Food exchange value

Relative Glycemic Impact of foods expressed as GBE represents the weight of bread that would induce a glycemic response equal to that induced by the given quantity of test food [3].

GBE for Chapatti and Marie biscuit is 44 which indicates that 50 g of these products produce similar glycemic response as that of 44 g of bread (approx 2 3/4 slices) while 50 g of Thepla induces glycemic response equivalent to that of only 17 g of bread (app. 1 1/3 slices) (Table 2).

The common serving sizes used in practice differs for each food item based on its structure, size, density and volume. Hence it is more relevant to express glycemic response in terms of food portions practically consumed by individuals. Since GBE values are expressed in gram units they can be used to predict probable responses to common serving sizes.

The approximate GBE values, when calculated for common serving measures, showed that consuming one serving of Thepla (2 small in number) is better than consuming chapatti (4 small or approx. 2 medium size) due to its lower predicted glycemic response (Table 2). Contrary to general notion, one serving of Marie biscuit (4 in number) as well as Chapatti (4 small/ 2 medium size) may produce increased hyperglycemia. This emphasizes the need to regulate the quantity to be consumed at one time for effective long term management of postprandial blood glucose.

From the point of view of postprandial metabolic response, Thepla seems to be the most favourable food compared to chapatti and Marie biscuit since it produces a lower glycemic and insulinemic effect. Addition of Bengal gram flour (protein), fenugreek (fiber) and fat in Thepla creates a balanced meal that can be easily consumed by the glucose intolerant as well as insulin resistant individuals without creating stress on the postprandial metabolism.

Table 2 Glycemic Bread equivalent for the standard and test foods

<table>
<thead>
<tr>
<th>Food product</th>
<th>HH* measure for 50gm cooked food</th>
<th>GBE /50 g of food</th>
<th>Bread equivalent (HH measure)</th>
<th>CSM*</th>
<th>Predicted GBE/ CSM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapatti</td>
<td>App.1 ½ med</td>
<td>43.8</td>
<td>2 3/4 slices</td>
<td>4 small (60 g)</td>
<td>53</td>
</tr>
<tr>
<td>Marie Biscuit</td>
<td>9 nos.</td>
<td>44.3</td>
<td>2 3/4 slices</td>
<td>4 in no (23 g)</td>
<td>20</td>
</tr>
<tr>
<td>Thepla</td>
<td>1 3/4 small size</td>
<td>17</td>
<td>app.1 1/3 slices</td>
<td>2 small (67 g)</td>
<td>23</td>
</tr>
</tbody>
</table>

*HH Household, CSM* Common Serving Measure
In conclusion, overall nutrient density and wholesomeness of the food product as consumed by end-user should govern food choices rather than drawing inference based on metabolic response to a single nutrient in food. Food products which do not cause prolonged hyperglycemia or hyperinsulinemia should be emphasized during counseling to the patients having insulin resistance, diabetes or at risk of cardiovascular disease. Food exchanges elucidated through Glycemic Bread Equivalent values will enable the consumer to choose foods and portion sizes that can provide balanced nutrition and facilitate long term blood glucose control. Further studies with larger sample size should be done and glycemic response to the test foods with common accompaniments should be tested to understand effect of mixed meals.

Acknowledgment  This study is supported through Doctoral Fellowship from Indian Council of Social Science Research (ICSSR) New Delhi. We are deeply grateful to the volunteers for their participation.

References