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Ameliorating effect of pomegranate peel extract supplement against type 1 diabetes-induced hepatic changes in the rat: biochemical, morphological and ultrastructural microscopic studies

Ameliorating effect of pomegranate peel extract supplement against hepatic changes in liver of diabetic rat

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Abstract

Background: Diabetes mellitus could result from disorders in insulin secretion or receptors mainly characterized by hyperglycemia. Natural antioxidants including pomegranate are traditionally used as hypoglycemic agents. The present research was designed to evaluate the possible therapeutic role of Pomegranate Peel Extract (PPE) against type 1 diabetic-induced hepatic biochemical and histological alteration.

Materials and methods: Adult male Wistar rats (N= 48) were sorted into four groups: G1: control group, G2: normal rats received PPE. G3: STZ-diabetic rats, received IP streptozotocin (55 mg/kg BW) and G4: Diabetic rats post-treated with PPE (200 mg/kg BW/day). Effectiveness of (PPE) was assessed by measuring serum glucose, liver enzymes, and morphological features of liver tissue using light and electron microscopy.

Results: Histological examination showed degenerative necrotic changes in diabetic rat liver which were improved by post-treatment with PPE. Biochemical results confirmed microscopic morphological and ultrastructural findings.
**Conclusions:** PPE was found to have a moderate therapeutic effect against a hepatic alteration in the male rats. It could be advised for diabetic patients suffering from the early alteration of liver functions.

**Key words:** pomegranate peel extract, therapeutic, streptozotocin, liver, diabetes, liver enzymes, ultrastructure

**INTRODUCTION**

Diabetes is a challenging clinical condition with many drastic complications on body organs (1, 2) including the liver (3). Hyperglycemia is the main feature of diabetes mellitus (4). It has many impacts on the structure and function of many organs (5, 6) including the liver (7, 8). Hepatocytes are well-known to be involved in the metabolism of different nutrients including carbohydrate (9).

Transmission electron microscopy (TEM) is used to give subcellular details of cells and define cell organelles involved in protein synthesis, energy production and lysosomal functions that can undergo alteration upon exposure to oxidative stress (10). Researches regarding hepatocyte alteration in diseases and therapeutic modulation were found in the literature. (11).

Alteration of mitochondrial electron transport chain which may be reflected in mitochondrial ultrastructure and their energy production was reported (12). Pomegranates is a famous fruit highly rich in antioxidants (13 .14) and its products were used as natural remedies for many diabetic oxidative - stress-induced lesions including liver (15). This study showed that Pomegranate juice significantly reduced hepatocyte lipid peroxidation and oxidative stress in type 2 diabetic rats through improving antioxidant status in liver tissue. Therapeutic uses of pomegranate were reviewed, and it was proved to be effective against many diseases such as dental infection, cardiovascular disease, and diabetes (16).

A study was done (17) proved its effective role in the amelioration of diabetic induced nephropathy in the rat.
The previous study by the author showed that PPE provided a prophylactic effect against diabetic induced changes in rat liver (18). Diabetes was reported to induce oxidative stress leading to tissue damage and this mostly occurs via alteration in mitochondria (19). Thus, in the present study Electron microscopy was used to confirm light microscopic morphological finding and describe mitochondrial and other organelles changes in the liver of diabetic rats besides demonstrating the therapeutic anti-diabetic effect of PPE together with confirming biochemical assay of liver enzymes alteration.

**Ethical approval**

This study was conducted according to guidelines and protocols approved by the ethical committee for animal care and use in King Fahd Medical Research Centre (KFMRC), KAU, Jeddah, Saudi Arabia, which are in accordance with the guidelines of the Canadian Council on Animal Care.

**MATERIALS AND METHODS**

**Drugs and chemicals:**

Pomegranate fresh fruit were obtained from Taif region (Al Bustan farm), Saudi Arabia. Methanol was purchased from Sigma-Aldrich, Chemie GmbH, Germany. Streptozotocin was obtained from Sigma- Aldrich Corp, St. Louis, MO, USA. Mouse ALT (Alanine Transaminase) ELISA Kit was obtained from Geno Technology, Inc. (USA). Rat Total Alkaline Phosphatase (TALP) ELISA Kit as well as Rat Aspartate aminotransferase (AST) ELISA Kit were purchased from My BioSource, Inc., California, San Diego (USA).

**Animals:**

Adult (3 months old) male Wistar rats (N=48) with an average body weight of 200-250 gm) were obtained from animal house,( animal care unit in King Fahd Medical Research Centre (KFMRC), KAU, Jeddah, Saudi Arabia,) according to the guidelines for animal research approved by the Unit of Biomedical Ethics Research Committee, Faculty of Medicine, King
abdulaziz University. Rats were divided into four groups (12 rats per cage) and allowed to adapt to lab conditions for one week, temperature 23 °C and 22 Co humidity with12H/12H light/dark cycle. They were fed with normal standard diet and water ad libitum.

**Methods**

**PPE preparation:**

Air-dried Peel was prepared from fresh pomegranate (Punica granatum). Dried peels (50gm) was pulverized and extracted for 24h using 500 ml absolute methanol using ULTRA-TURRAX disperser (T 50 basic IKA-Werke, Germany). The resulted extract was filtered and evaporated using rotary evaporation (Rotavap, BUCHI, Switzerland). The extract was kept at 20 °C.

**Diabetes induction:**

STZ (55mg/kg BW) was dissolved in 0.05 M citrate buffer (one ml) at pH 4.5 and injected immediately after preparation through intraperitoneal route to fasting rats. Glucose (5%) was given in drinking water to overcome STZ induced hypoglycemia).

Three days later, blood glucose from fasted rats was taken from tail vein then measured using glucometer. Animals having blood glucose ranged from 300-500mg /dl were chosen as a model for type1 diabetes and included in the experimental procedure (20).

**Animal grouping and study design**

The experimental procedure last for 12 consecutive weeks where rats were divided into 4 groups:

**G1**: served as the control, **G2**: rats received an oral dose of PPE (200 mg/kg BW/day). **G3**: STZ- diabetic rats, **G4**: Diabetic rats administrated PPE in a similar dose to G2.

The following parameters were recorded:
Body weight
The body weight of all animal groups was recorded at the end of the experiment before sacrificing and was statically analyzed.

Blood glucose:

For evaluation of treatment response fasting glucose level was measured weekly all through the experiment using a blood glucometer (ACCU-CHEK; Roche Mannheim, Germany). Blood glucose levels measured for all groups at the end of the experiment and were subjected to statistical analysis.

Biochemical study for liver enzymes:

Blood samples were collected from the retro-orbital venous plexus lightly anesthetized animals before animal sacrifice. Serum was obtained by centrifugation at 4 °C, 3000 rpm for 10 min then stored at -20°C for liver enzyme (ALT, AST, ALP) analysis (21). The analysis was done in Mansour Scientific Foundation for Research & Development, Jeddah, Saudi Arabia.

Tissue processing for morphological and ultrastructural microscopic features:

The rats were anesthetized lightly by diethyl ether inhalation (one ml in soaked cotton pellets) and the abdominal cavity was incised at the midline. The liver was removed and samples from the large lobe (2X2mm) were fixed either in 10% neutral buffered formalin or in 3%glutaraldhyde in phosphate buffer pH 7.4 at 4 °C. Routine processing for light paraffin sections and electron microscopy was carried out in highly specialized lab in KAU hospital and electron microscopy unit. Paraffin sections are stained by hematoxylin and eosin while semi-thin sections (0.5-1 mm) were stained with 1% toluidine blue for general orientation using light microscope. Ultrathin sections (60 nm thick) were done by ultra-tome then processed for staining using 2% uranyl acetate and lead citrate (22). Photographing of ultrathin stained sections was done using a transmission electron microscope. (80 kV, JEM-100 Cx11, JEOL) in an electron microscopic unit. Assuit University, Egypt.

Statistical analysis
Statistical analysis using "IBM SPSS Statistics ver. 20.0" was applied to evaluate and test the hypothesis. The results were presented as means ± standard deviations (SD). One-way analysis of variance (ANOVA) was used to find the significant differences between the four groups’ means followed by a post hoc test, Tukey HSD for multiple comparisons. Results were considered statistically significant when p < 0.05.

RESULTS

I. Body weight changes:
Table (1) showed the body weight of G3(STZ) was decreased significantly (P < 0.01) as compared with G1 (control) and G2 (PPE). The rats in G4 (STZ + PPE) showed an increased in body weight as compared with G3. PPE did not alter body weight of control rats. However, it was observed that the decrease in body weight in the diabetic rat was restored by PPE administration.

II. Fasting Blood glucose (FBG) alteration:
Table (1) showed that fasting blood glucose of G3 was increased significantly (P < 0.01) as compared with control G1, and G2. The rats in G4 showed a decrease in FBG as compared with G3. PPE was found to lower blood glucose of diabetic rats but insignificantly compared to control. PPE, on the other hand, did not alter the blood glucose of control animals.

III. Liver enzymes (ALT, AST, ALP) serum levels alteration:
Table (1) showed the liver enzymes level in G3 was increased significantly (P < 0.01) as compared with control G1, and G2. The rats in G4 showed decreased in liver enzymes as compared with G3.
Liver enzymes were assayed in this study to demonstrate any hepatocyte cell injury or necrosis that may lead to the release of enzymes into blood circulation.

Table 1. Effects of STZ and Pomegranate Peel Extract on body weight, blood glucose, and liver enzymes in rats.
<table>
<thead>
<tr>
<th></th>
<th>G1 (control)</th>
<th>G2 (PPE)</th>
<th>G3 (STZ)</th>
<th>G4 (STZ + PPE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose (mg/dl)</td>
<td>91.89394±3.54</td>
<td>97.12963±4.72</td>
<td>464.9583±26.45</td>
<td>392.8485±9.67</td>
</tr>
<tr>
<td>Alanine aminotransferase (ALT) (U/L)</td>
<td>23.16667±7.34</td>
<td>26.83333±7.67</td>
<td>56.5±7.65</td>
<td>41.83333±6.62</td>
</tr>
<tr>
<td>Aspartate aminotransferase (AST) (U/L)</td>
<td>54.33333±12.23</td>
<td>54.66667±9.30</td>
<td>154.8333±26.62</td>
<td>78±15.52</td>
</tr>
<tr>
<td>Alkaline phosphatase (ALP) (U/L)</td>
<td>100.5±14.68</td>
<td>80.66667±9.45</td>
<td>209.5±54.70</td>
<td>146.6667±9.83</td>
</tr>
</tbody>
</table>

The number of animals was 12 for each group, all values are expressed as means ± SD.

aSignificance versus G1 (control); bSignificance versus G3 (STZ); (p < 0.05)

Histopathological study

I. Effects of diabetes type 1 and PEE treatment on the morphology of liver tissue using light microscopy:

Fig. (1) showed photographs from the light microscopic examination of paraffin (H&E stain) rat liver sections from control group 1 rats (Fig. 1a). PPE extract group (1b), diabetic STZ group (1c) and diabetic STZ-PPE group (1d) treated rats.

In (Fig 1a), hepatocytes were found to be arranged radially around the central vein. Cells have rounded central vesicular nuclei and showed an acidophilic cytoplasm. Thin-walled blood
sinusoids were observed between hepatocyte cell cords. They are lined by flat endothelial cells. Occasionally, Kupffer cells nuclei could be seen in some sinusoids. In (Fig 1b), showed no alteration of normal structure. In (Fig 1c), the liver showed scattered apoptotic hepatocytes (smaller in size than normal cells and have deeply stained acidophilic cytoplasm and small dark nuclei). Some samples showed necrotic cells with ill-defined outlines. In (Fig 1d) marked improvement was observed where the liver showed nearly normal radially arranged hepatocytes around the central vein. Blood sinusoidal spaces and their Kupffer cells also looked like control

II. Effects of diabetes type 1 and PEE treatment on the morphology of liver tissue using electron microscopy:

a. Semithin sections (toluidine blue stain).

The technique of semithin sectioning provided more detailed features of liver parenchyma. In control animals, Hepatocytes with polyhedral shapes are normally arranged around the central vein were observed. The cells have vesicular nuclei with prominent one or two nucleoli. Blood sinusoids between hepatocytes are lined by endothelial and Von-Kupffer cells (Fig. 2a).

In (Fig. 2b) no alteration was observed. Alterations by STZ -induced diabetes were shown in (Fig.2c) Marked damage of CV endothelial cell lining was observed. Hepatocytes appeared shrunken. Their nuclei looked smaller, darker and degenerated, Blood sinusoids are dilated. Nearby hepatocytes showed necrosis leaving empty spaces.

In (Fig.2d) Hepatocytes showed normal rounded central nuclei and prominent nucleoli, the cytoplasm of some cells dark stained granules. Blood sinusoids between the cells are thin.

b. Ultra-structural changes in rat hepatocytes.

The Ultra-structural study was used to confirm what was seen by light microscopy as it showed cell organelles changes in all experimental groups compared to control.

Electron microscopy micrographs from control liver (Fig. 3. a, aa) showed that hepatocytes have a normal population of mitochondria, rough endoplasmic reticulum and glycogen granules with few fat globules. The nuclei showed an euchromatic appearance.
Blood sinusoids are lined by normal Von-Kupffer cells.

In (Fig. 3. b) there was no alteration in ultrastructure features of hepatocytes.

In (Fig. 3c (a), 3c(aa)). Electron microscopic photographs of diabetic rat hepatocytes showed enlarged nuclei called karyomegaly. Binucleated hepatocytes with double nuclei were observed., Hepatocyte cytoplasm showed focal regions of organelle degeneration, few swollen mitochondria, lipid droplets, and dark lysosomal bodies. In (Fig. 3. d) where diabetic rats were treated with PPE, ultrastructure features of hepatocytes showed absence of changes seen treated diabetic group. The nucleus is normal. The cytoplasm contains rough endoplasmic reticulum beside numerous normal mitochondria. Smooth endoplasmic reticulum (SER) beside few cytoplasm vacuoles were observed.

DISCUSSION

The liver is considered the largest important organ that deals with metabolic processing of many nutrient materials including carbohydrate beside toxin and drug detoxification in both normal and diseased conditions (23). Diabetes is associated with the increased process of oxidative stress induced by hyperglycemia (24). The increased free radicles in oxidative stress damaged many organs including the liver (25, 26).

Light microscopic examination in the present study showed alteration in diabetic rat liver in the form of disordered hepatic cords, appearance of scattered apoptotic cells, dilation in central vein and sinusoids, damaged lining of central veins. Similar results were reported by, (27, 28, 29). STZ-cytotoxicity on pancreatic islet β-cells resulting in hyperglycemic status that interferes with cellular metabolic oxidative mechanisms (30). Animal diabetic models exhibit high oxidative stress due to chronic hyperglycemia that results in depletion of the antioxidant defense system and promotes de novo generation of free radicals (31).

In the present study, semithin sections beside ultrastructural features of normal rat hepatocytes reflected what was seen in H&E stained paraffin. In EM micrographs the central vesicular
nuclei appeared euchromatic where chromatin was seen dispersed in the nucleoplasm with prominent nuclei. Such appearance indicated normal cellular activity (32). On the other hand, hepatocytes from diabetic rats showed altered ultrastructure in the form of nuclear chromatin condensation (heterochromatic appearance), loss chromatin condensation (heterochromatic appearance), loss and damage of rER which appeared as electron-lucent regions with reticulated appearance. There was also a decreased mitochondria population. A similar observation was seen in many cases of hepatocyte cell injury in toxic conditions (24). In diabetic status, similar ultrastructure alteration of hepatocyte in case of alloxan-induced diabetes in rat liver were described (11).

Loss of endoplasmic reticulum and decreased mitochondria population in hepatocytes of STZ-induced type 2 diabetes were also reported (33). Also, Farid, et al. (34) described disorganization and degenerative changes in hepatocyte cytoplasmic organelles of diabetic rats.

Mitochondria integrity is critical for cellular health. In the present study, hepatocyte mitochondria showed a marked decrease in G3 diabetic rats. The decrease in mitochondria may affect energy production with subsequent derangement of functional activity. Hyperglycemia was reported to cause damage of mitochondrial structure and function in tubular and mesangial cells of the diabetic rat kidney (19).

In a study done by Chang et al. (35), hyperglycemia was found to affect mitochondrial replication and fusion needed to face nutrient depletion (35). Hyperglycemia was reported to increased Ca2+ within mitochondria and this may cellular function (36). Mitochondrial integrity and function were affected in the case of Hepatic insulin resistance (37).

Accumulation of lipid droplets and lysosomal bodies in hepatocytes of the diabetic rats was demonstrated in this study at the level of EM. Similar observations were reported using HepG2 cells and was attributed to alteration of enzymes (inducible kinase) involved in the process of lipogenesis (38, 39). Hyperlipidemia associated with type 2 diabetes mellitus results in lipid deposition in different tissue including the liver (40, 41). Steatosis or hepatic liver accumulation of lipids was also described in detail in the rat model (42).
Dense bodies observed in rat hepatocytes of G 3: diabetic rats were most probably lysosomal structures that were previously described and associated with hepatocellular autophagy in alloxan-induced diabetes in male mice (43). The increase in the lysosomal structure was attributed to oxidative stress and increased free radical formation in diabetic status (44, 45).

In animals receiving PEE, hepatocytes showed the absence of changes induced by diabetes and looked similar to those of control.

Ultrastructure features of rat hepatocytes showed less features of necrosis, apoptosis, Bioactivities of pomegranate peel extract as an antioxidant was reported by (46). It was used for the protection of many organs as in case diabetic nephropathic changes (17) and was used to antagonize oxidative stress induced by diabetes (47). Diabetic rats were given extracts of Pomegranate peel (Punica granatum) showed improved diabetic status (48). The effect was reported by the authors to be via increasing antioxidants enzymes that antagonize oxidative stress of diabetic status.

Hepatic steatosis in diabetic patients was reported to be prevented by Ellagic acid, the active ingredient of pomegranate (49). Most previous researches regard pomegranate products deal with preventive activities against diabetic induced hepatic changes (50) This study, however, proved its potential therapeutic effect against diabetic changes in liver parenchyma.

However, an updated review about the health benefit of pomegranate products including PPE was done by Vučić, et al. (51) who reported that more investigations have to be done to clarify the mechanism of its antidiabetic action before use as a therapy.

CONCLUSIONS

It could be concluded that morphological features of liver tissue using both light and ultrastructural microscopic studies provided an idea concerning cellular and subcellular changes in case of diabetes compared to control. Mitochondrial alteration observed here confirmed that diabetic changes are due to oxidative stress. which explained the improvement occurred via administration of PPE; the natural supplement that proved to possess high antioxidant activity. Further studies on the same samples are running to confirm this
mechanism. More work must be done via clinical trials to evaluate its beneficial effect in controlling diabetic hyperglycemia and its complication especially hepatic in humans.

Acknowledgement

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Legends of figures:

Fig. (1) Photomicrographs of transverse sections of rat liver. Magnified power (1000X) stained by H&E to show:

a. G1: (control). Part of central vein (CV) with intact endothelial lining (dotted arrows). Hepatocytes cell cords with rounded vesicular nuclei (black arrows) and slightly basophilic cytoplasm. Blood sinusoids are of normal appearance (white arrows)
b. G2: (PPE). No alteration in liver histology, central vein (CV) is lined by intact endothelium (dotted arrow). Normal hepatocytes with normal nuclei (black arrows) and blood sinusoids (white arrows).
c. G3: (STZ) diabetes. Showing damage of CV endothelial cell lining (dotted arrows). Hepatocytes are shrunken with their nuclei looked smaller darker and degenerated (black arrows) all are features of apoptosis. Many hepatocytes are lost leaving necrotic regions (stars)
d. G4: (STZ+PPE). Liver parenchyma with both hepatocytes (black arrows) and blood sinusoids (white arrows) looked normal and similar to control.

Fig (2) Photomicrographs of Semithin sections from rat liver. Magnified power (1000X) stained by toluidine blue to show:

a. G1: (control) Notice the normal hepatocyte with their central rounded vesicular nuclei with prominent nucleoli (black arrows). Thin wall blood sinusoids with blood cells could be seen among hepatic cell cords (white arrows).
b. G2: (PPE) showing also normal hepatocytes with rounded vesicular nuclei (black arrows). Blood sinusoids showed prominent Vonkupffer cell nuclei (white arrow).
c. G3: (STZ) Marked damage of CV endothelial cell lining (dotted arrows). Apoptotic hepatocytes are shrunken with their nuclei looked smaller, darker and degenerated (black arrows). Blood sinusoidal lumina are dilated (white arrow). Many hepatocytes are lost leaving necrotic regions (stars)
d. G4: (STZ+PPE) Showing hepatocytes with normal rounded central nuclei and prominent nucleoli (black arrows). The cytoplasm of some cells showed dark stained granules. Blood sinusoids between the cells are thin (white arrows).
Fig (3) Electron micrographs of ultrathin sections from hepatocytes showing:

G1: control

a. Low magnification (3600 X) to show hepatocyte and part of nearby sinusoids lined by Von Kupfer cells (white arrow). Hepatocytes nucleus (N), Mitochondria (M), rough endoplasmic reticulum (white arrows). smooth endoplasmic as small vesicles (dotted arrows).

aa. Higher magnification (5800 X) to show details of hepatocyte organelles and nucleus

b. G2: (PPE) showing binucleated hepatocyte (N) with prominent nucleoli (n). the cytoplasm showed numerous mitochondria (M), rough endoplasmic reticulum (white dotted arrows) and few small dense lysosomal bodies (black arrows). (5800 X)

c. G3: (STZ) showing hepatocytes from diabetic rat liver

a. hepatocytes with enlarged nuclei called karyomegaly(N). focal area of cell organelle degeneration. Cytoplasm contain few swollen mitochondria (M), lipid droplets (White arrows), dark lysosomal dense bodies (dotted arrows). (5800X).

aa. Binucleated hepatocytes with double nuclei(N). One showed peripherally located nucleolus(n). Cytoplasm showed regions of organelle degeneration (white stars). Lysosomal bodies (dotted arrows) and mitochondria(M). (3600X).

d. G4: (STZ+PPE) showing absence of changes seen in pomegranate treated diabetic group (STZ+PPE), the nucleus is normal (N) The cytoplasm contains rough endoplasmic reticulum (white arrows) numerous normal mitochondria (M). Few vacuoles (black arrows). Notice, Regions of smooth endoplasmic reticulum (SER). (5800).