

GREEN TEA MITIGATES STREPTOZOTOCIN-INDUCED DIABETIC MICE THROUGH ANTI-FIBROTIC ACTIVITY AND MODULATION OF PROINFLAMMATORY CYTOKINES

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Abstract: This research was planned to assess the protective effect of green tea extract (GTE) in STZ-induced diabetes in mice. Seventy-five female albino mice were used. Mice randomly allocated into five groups of 15 mice in each as follows: group 1 (control), group 2 (STZ, diabetic), group 3 (green tea + STZ), group 4 (protective group with green tea), and group 5 (green tea only). Oral administration of the green tea extract for three weeks to diabetic mice resulted in significant regaining in body weight, decreased blood glucose, cholesterol, triglyceride, free fatty acid and low-density lipoprotein cholesterol (LDL-C) levels and lowered malondialdehyde (MDA) contents and increased total protein and high-density lipoprotein cholesterol (HDL-C) as compared to untreated diabetic group. Histopathological changes were compatible with these biochemical findings. Diabetic mice pre- or co-treated with green tea also exhibited a significant downregulation in *TGFβ1* and *TNFα* genes expression. Green tea extracts had protective and antidiabetic effect in controlling blood glucose level in addition to improving lipid metabolism and body weight in diabetic mice.

Key words: diabetes; green tea; liver; TNFα; TGF-B1; mice

Introduction

Diabetes mellitus (DM) is one of the major intimidations to health in the world. It was expected to be more prevalent in 2025 (1). Hyperglycemia is associated with vascular complications and kidney diseases (2). There are several other factors that play a great role in diabetes for instance, obesity and oxidative stress which lead to more ferocious complications (3), with decreased humoral immunity (4).

DM type 1 is a consequence of the annihilation of β-cells of the pancreas (5). Deficiency of insulin primes to hyperglycemia. STZ is a structural analog of glucose (Glu) and N-acetyl glucosamine is reserved up by β-cells via the GLUT 2 transporter subsequently lead to β-cell loss via DNA destruction (6, 7). Moreover, cells that express GLUT 2 transporter like liver and kidney cells are also vulnerable to STZ. This clarifies kidney and liver complications (8, 9).

Oxidative anxiety is an inequity among the reactive oxygen species (ROS) and antioxidant protection of the body, which play a great role in diabetic complications (10). Since many studies stated that oxidative anxiety, facilitated by diabetes-induced production of ROS, lead to progress of diabetes, it noticed that improving oxidative anxiety via antioxidants might be a successful approach for demoting diabetic complications (11, 12).

Green tea (*Camellia sinensis*) is prevalent nutraceutical antioxidant especially in Asian countries (13). Green tea has an important role in decreasing blood pressure, low-density lipoprotein cholesterol, and oxidative stress (14). Many studies proved the valuable effects of green tea on diabetic complications (15, 16) as well as it has antihyperglycemic effect in STZ diabetic animals (17). Moreover, green tea shows antioxidants and free radicals scavenger properties (18).

This study was done to evaluate the protecting pathway by which green tea alleviate streptozotocin-induced diabetes in mice.

Material and methods

Chemicals

We utilized a pharmaceutical-grade of STZ from Sigma (St. Louis, MO, USA). The STZ solution prepared by dissolving STZ in citrate buffer (0.01 M, pH 4.5) (19, 20). Chemicals consumed were of analytical grade. Total cholesterol, high-density lipoprotein (HDL)-cholesterol and triglyceride (TG) standard kits were purchased from Erba Diagnostics Mannheim GmbH, Germany.

Green tea extract

The green tea tablets, each tablet contain green tea extract 200 mg manufactured by El Obour City pharmaceutical industries. Following grinding, the obtained green tea powder dissolved in distilled water. This solution was provided to mice orally by using a stomach tube.(21)

Experimental animals

Seventy-five female albino mice, (9 – 11 weeks old, weighing about 25-30 gm was used.

They obtained from Medical Technology Center, University of Alexandria, Egypt. Animals were managed according to the rules and regulations of our university committee. Female albino mice were housed in stainless steel pens of ambient temperature $23\pm 2^{\circ}\text{C}$ and light (12 h light/12 h dark) and free access to food and water. The mice were fed a standard diet according to (22) .

Initiation of experimental diabetes

After 2 weeks of accommodation, the mice were exposed to a 12 h fast. The mice were I/P injected with a single dose of 200 mg/kg STZ (7, 19). STZ was freshly dissolved in 0,1M cold sodium citrate buffer, pH 4.5(19, 20).after 2h from injecting we put 5% glucose and food to injected mice to avoid death(20) .we replace 5% glucose with water after 2h.The urine glucose level was checked three days after STZ injection to check the progress of diabetes. The diabetic mice were steadied for 5 days and then the experiment beginning. Mice which showed glucose levels >250 mg/dL was considered diabetic.

Experimental design

After acclimatization, mice were alienated randomly into five groups of 15 mice each: Group 1: healthy control mice received distilled water Group 2 (diabetic group): will be used as control diabetic mice. The mice were injected I/P with one dose of 200 mg/kg STZ (7, 19). In group 3 (treated group with green tea) diabetic mice were administrated green tea at a dose of 200 mg/kg bw orally by stomach tube daily for 21 days. In group4 (protective group with green tea) mice were given green tea at dose of 200 mg/kg by stomach gavage for 21 days and then diabetes was induced with a single dose of 200 mg/kg streptozotocin with continuous treatment with green tea at dose of 200 mg/kg orally for 21days. In group 5 (control green tea group) non-diabetic mice received green tea (200 mg/Kg /day)dissolved in distilled water orally by stomach tube for 21 days according to (23).

Sampling

Mice were sacrificed by cervical decapitation. Body weights of all the animals were recorded prior to the treatment and sacrifice. Weight change % = $\frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100$

Blood samples were collected in either EDTA coated tubes for the hematological investigation or plain tubes for serum separation (24) for biochemical assays. Kidneys, liver, spleen, and pancreases after decapitation were quickly removed and washed by cold saline to remove extraneous materials. Each organ was divided into 3 specimens. The first was snap frozen with liquid N₂ and kept at -80°C until use for RNA extraction. The second was fixed in 10% neutral buffered formalin for the histological examination. The third was used for transmission electron microscopy (TEM) handled using standard techniques.

Blood analysis

The serum samples for measurement of blood glucose level was determined based on glucose oxidase method (25), insulin concentrations were determined according to (26) using an insulin-ELISA kit (Morinaga Seikagaku, Tokyo, Japan). Malondialdehyde (MDA) was quantified by the method of Ohakawa et al. (27), HbA1c was appraised using DCA 2000 analyzer (Bayer, Elkhart, IN)(28, 29). Total protein concentration in serum was assayed colorimetrically using commercial kit (Diamond, Egypt) and according to Lowry et al. (1951). Serum levels of total cholesterol, triglycerides, and high-density lipoproteins cholesterol (HDL-C) were estimated by quantitative colorimetric assay (Stanbio Laboratory, Inc., Texas, USA) according to (30) and low-density lipoprotein cholesterol (LDL-C) concentration assessed according to the formula of Friedewald et al; (31). LH, FSH, and estrogen were assessed using IMMULITE chemiluminescent assay kits (DPC, Glyn Rhonwy, Llanberis, Gwynedd, UK)(32). The Horiba ABX 80 Diagnostics (ABX pentra Montpellier, France) was used for hematological examination (33).

Histopathological and transmission electron microscope examination

The histopathology was carried out according to (34) using hematoxylin and eosin staining technique. Preparation of samples for transmission electron microscope (TEM) using collagen-coated nickel grids was applied as previously described (35).

Molecular investigation

Total RNA was extracted from tissue samples using RNA extraction kit (easy-REDTM, iNtRON Biotechnology, #17063, South Korea). cDNA synthesis was performed using reverse transcription kits (Thermo Scientific, Fermentas, #EP0451) which include the following: Revert Aid H Minus Reverse Transcriptase enzyme, Oligo dT, 5X Reaction Buffer, RiboLock RNase Inhibitor, and dNTP Mix. Concentration of RNA and cDNA quantify using a Nanodrop (Q5000 Uv-Vis spectrophotometer, USA).

Real-time PCR with SYBR Green (2X Maxima SYBR Green/ROX qPCR Master Mix) used to determine the expression of the target genes in the liver, with GAPDH as a house-keeping gene, according to the manufacturer protocol (Thermo Scientific, USA, # K0221). The primers used in the amplification are shown in Table 1. The web-based tool, Primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) used to design these primers. To confirm primer sequence is unique for the template sequence; we check similarity to other known sequences with BLAST. Calculation of fold changes in gene expression was done using $2^{-\Delta\Delta C_t}$ method.

Statistical analysis

Data was analyzed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Results subjected to Tukey's multiple comparisons post-hoc test. Values are statistically significant when $p < 0.05$.

Results

Growth weight and Biochemical analysis

Diabetic mice had a significant drop in body weight in relation to other groups as shown in

figure 1. The diabetic mice showed a significantly increased level of serum glucose, triglycerides, cholesterol, LDL-C, VLDL-C relative to the control group and other treated groups and green tea protective groups. Serum insulin, total protein and HDL-C statistically decreased in diabetic mice as compared to the control group, treated and green tea protective groups (Table 2). Serum HbA1C pointedly increased in diabetic mice relative to the control group (Table 2). Diabetic mice showed a noteworthy increase in MDA compared to their equivalent levels in control animals. However, treated groups with green tea showed a significant diminution in the levels of MDA in relation to diabetic mice. Protective groups treated with green tea exhibited a significant reduced MDA levels as compared to diabetic mice (Table 2). LH, FSH and estrogen levels did not show any significant difference among all groups (Table 3).

Hematological analysis

RBC and WBC count, PCV, neutrophil % and platelets levels in diabetic mice decreased significantly, while MCV, MCH, and MCHC increased significantly in diabetic mice compared to the control mice. Green tea extract significantly normalized RBC and WBC count, PCV, neutrophil % and platelets in diabetic mice as well as MCV, MCH, and MCHC of diabetic mice to the control level (Table 4).

Histopathological studies

All results of histopathology were displayed in figure 2. Histopathological observation revealed that liver of the control animal showing normal hepatocytes arranged in cords around the central vein (arrowhead) whereas that of STZ-treated animal showing single cell necrosis (arrowhead) associated with active apoptosis (arrow). Green tea treated diabetic animal showing slight hepatic vacuolation (arrowhead) adding that liver of green tea group showing normal hepatocytes around the central vein (arrow) as well as liver of protective group pretreated with green tea showing normal hepatocytes arranged around the central vein (arrow). Kidney of control animal showing normal renal glomeruli (arrowhead) and tubules (arrow)

while kidney (cortex) of STZ-treated animal showing marked glomerular congestion (arrowhead) and degeneration within renal tubules (arrow) while that of green tea treated diabetic animal showing normal renal glomeruli (arrowhead) and patent normal tubules (arrow). Green tea group showing normal renal glomeruli (arrowhead) and tubules (arrow). Kidney of protective group with green tea-treated animal showing renal glomeruli (arrowhead) and tubules (arrow) within the normal limits. Pancreas of control animal showing normal glandular acini (arrow) and β islets (arrowhead) while that of STZ-treated animal showing degeneration of both glandular acini (arrow) and β islets (arrowhead). Green tea treated diabetic animal showing normal glandular acini (arrow) and slight vacuolation of β -cells (arrowhead) as well as pancreas of green tea-treated animal showing normal glandular acini and β islets (arrowhead). Diabetic animal pre-and post-treated with green tea-treated animal showing normal glandular acini (arrow) and β cells (arrowhead).

Electron microscope studies

All results of transmission electron microscope were displayed in figure 3. Liver of control group showing normal hepatocytes M indicates mitochondria and N indicates nucleus. While that liver of diabetic group showing multilocular cytoplasmic fat vacuoles, decreased mitochondrial number and shrinkage of nuclear membrane. As well as, liver of green tea treated diabetic animals showing presence of small fat vacuoles, glycogen granules and multiple autophagic vacuoles. Besides green tea group showing three adjacent hepatocytes separated with thin connective tissue layer and mostly normal. M indicates mitochondria, bar=500 μm ., liver of protective group treated with green tea group showing moderate degree of hepatic steatosis, F indicates fat vacuoles. Pancreas (endocrine portion) of control group showing presence of large number of B secretory granules within the B cells (arrowhead), BC indicates blood capillary, pancreas of diabetic group showing exocrine pancreatic cell with noticeable decrease the number of secretory granules

and mostly immature (arrowhead) with corrugated nuclear membranes (arrow), while that Green tea treated diabetic group showing exocrine pancreatic cell revealing normal nucleus (N) and with normal network of rough endoplasmic reticulum (RER), pancreas of green tea group showing two adjacent exocrine cells with normal nuclei and surrounded with network of rough endoplasmic reticulum, in addition, pancreas (exocrine cell) of protective group pretreated with green tea showing normal nucleus surrounded with network of rough endoplasmic reticulum and revealing large number of mature secretory vesicles (arrowhead). kidney of control group showing normal lining epithelial cells of the distal renal tubules with normal mitochondria (M) with mild condensation. Adding, kidney of STZ treated group showing the lining epithelial cells of the proximal tubules with slightly condensed mitochondria and some of them showing presence of fat vacuoles (arrowhead) and with normal brush border covering (arrow). as well as, green tea treated diabetic group showing lining epithelial cells of the distal renal tubules and mostly revealing lysis of the most intracellular organelles (arrow), kidney of green tea group showing the proximal renal tubules lining epithelial cells with normal brush border and mitochondria (M), bar=500 μ m., while that of protective group pretreated with green tea group showing lining epithelial cells of the distal renal tubules and mostly revealing lysis of the most intracellular organelles (arrows).

Outcome of streptozotocin, green tea on the relative expression of $TGF\beta 1$ gene and $TNF\alpha$ gene

The results of qPCR were presented in Figure 4. A considerable upregulation in the expression of $TGF\beta 1$ gene in liver was found following administration of Streptozotocin as compared to the control group and other treated groups. Green tea treated diabetic mice showed a noteworthy decrease $TGF\beta 1$ gene expression as compared to the diabetic group. Furthermore, pre-treatment by green tea led to a significant decrease in $TGF\beta 1$ expression. Green tea alone led to insignificant downregulation of $TGF\beta 1$ gene expression as compared to the control group. $TNF\alpha$ upregulation was found following administration of Streptozotocin as matched to normal one and other treated groups. Administration of green tea with Streptozotocin resulted in a significant decrease in $TNF\alpha$ gene expression as compared to diabetic one. Furthermore, pre-treatment by green tea led to a significant reduction of $TNF\alpha$ expression. Administration of green tea alone led to momentous decreased of $TNF\alpha$ gene expression in relation to control group.

Table 1: Sequences of primers used in qPCR

Gene	Reverse primer (/5 ----- /3)	Forward primer (/5 ----- /3)	Size (bp)	Accession number
$TGF\beta 1$	GACGTCAAAGA- CAGCCACTCA	GCAACATGTGGA- TACCAGA	106	M13177
$TNF\alpha$	CTGATGA- GAGGGAGGCCATT	GCCTCTTCTCAT- TCTTG	115	NM_00127860 1
GAPDH	CCTGCTTCACCACC TTCTTGA	TGTGTCCGTCGTG- GATCTGA	99	M32599

Table 2: Effect of green tea on serum glucose, HbA1C, insulin, MDA, total protein levels in STZ-induced diabetic female albino mice

	Control	STZ	STZ+Green tea	pretreated with green tea	Green tea
Glucose (mg/dl)	120.5±13 ^c	407±49.6 ^a	186±27 ^b	115±23 ^c	143±4.7 ^{bc}
HbA1C (%)	5.00±0.0 ^c	13.0±0.44 ^a	8.2±0.58 ^b	5.0±0.0 ^c	8.2±0.58 ^b
Insulin pg/ml	1024±0.0 ^a	299.0±0.0 ^c	606±21.7 ^b	1024±0.0 ^a	826±50.8 ^b
MDA nmol/ml	0.557±0.0 ^c	1.90±0.0 ^a	1.0±0.06 ^b	0.57±0.0 ^c	0.8±0.01 ^b
Total protein (mg/dl)	6.21±0.17 ^a	4.44±0.18 ^c	6.175±0.4 ^a	6.14±0.12 ^a	5.30±0.46 ^b

Data are expressed as mean ± SEM. Superscript of different letters in each column differ significantly (P<0.05) from each other

Table 3: Effect of green tea on LH, FSH and estrogen level levels in STZ-induced diabetic female albino mice

	Control	STZ	STZ+Green tea	pretreated with green tea	Green tea
Estrogen(pg/ml)	24.2±0.3	22.8±0.5	23.0±0.4	23.6±0.24	22.8±0.37
FSH (pg/ml)	0.18±0.0	0.17±0.0	0.15±0.00	0.18±0.0	0.17±0.0
LH (pg/ml)	0.36±0.0	0.36±0.0	0.35±0.0	0.36±0.0	0.36±0.0

Data are expressed as mean ± SEM. Superscript of different letters in each column differ significantly (P<0.05) from each other

Table 4: Effect of green tea on Haemogram in STZ-induced diabetic female albino mice

	Control	STZ	STZ+Green tea	pretreated with green tea	Green tea
RBC (10 ⁶ /ul)	8.88±0.6 ^a	7.45±0.13 ^c	8.31±0.18 ^b	8.6±0.04 ^{ab}	8.07±0.05 ^b
Hb (g/dl)	15.4±0.18 ^a	12.9±0.24 ^c	14.5±0.26 ^a	14.3±0.21 ^b	14.5±0.2 ^{ab}
HCT %	29.48±0.3 ^c	37.0±0.42 ^a	31.2±0.33 ^b	31.0±0.48 ^b	29.2±0.20 ^c
MCV(fl)	40.7±0.28 ^c	46.0±0.28 ^a	43.0±0.35 ^b	43.1±0.23 ^b	40.7±0.35 ^c
MCH(pg)	13.2±0.09 ^c	17.5±0.21 ^a	14.1±0.13 ^c	13.9±0.05 ^c	13.65±0.1 ^c
MCHC (g/dl)	30.3±0.23 ^c	41.4±0.25 ^a	33.4±0.34 ^b	32.83±0.1 ^b	31.1±0.13 ^c
WBC (10 ³ /ul)	17.0±0.07 ^a	3.54±0.39 ^c	7.07±0.22 ^b	7.82±0.44 ^b	6.15±0.42 ^c
Lymphocyte %	65.0±0.16 ^a	11.94±4.1 ^c	45.65±4.1 ^b	44.59±3.3 ^b	61.21±0.6 ^a
Monocyte %	17.8±0.11 ^a	5.345±0.4 ^c	14.95±0.0 ^b	13.83±0.2 ^b	17.08±0.1 ^a
Granulocytes %	70.1±2.50 ^a	29.48±1.5 ^c	52.00±1.5 ^b	56.18±1.3 ^b	69.75±1.9 ^a
Platelets (103/ul)	637.5±54 ^a	349.3±17 ^c	422±13.9 ^{bc}	543.0±13 ^b	486±14.6 ^b

Data are expressed as mean ± SEM. Superscript of different letters in each column differ significantly (P<0.05) from each other

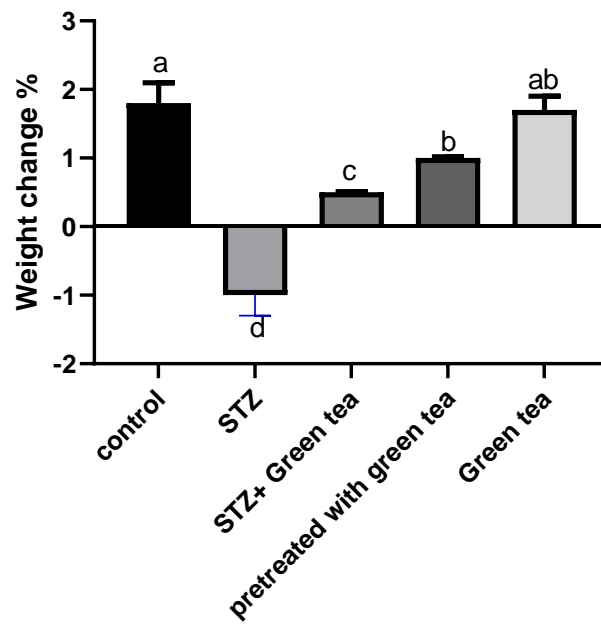


Figure 1: Effect of green tea on body weight, food intake and water intake grade in STZ-induced diabetic female albino mice. Data are expressed as mean \pm SEM. Superscript of different letters in each column differ significantly ($P < 0.05$) from each other

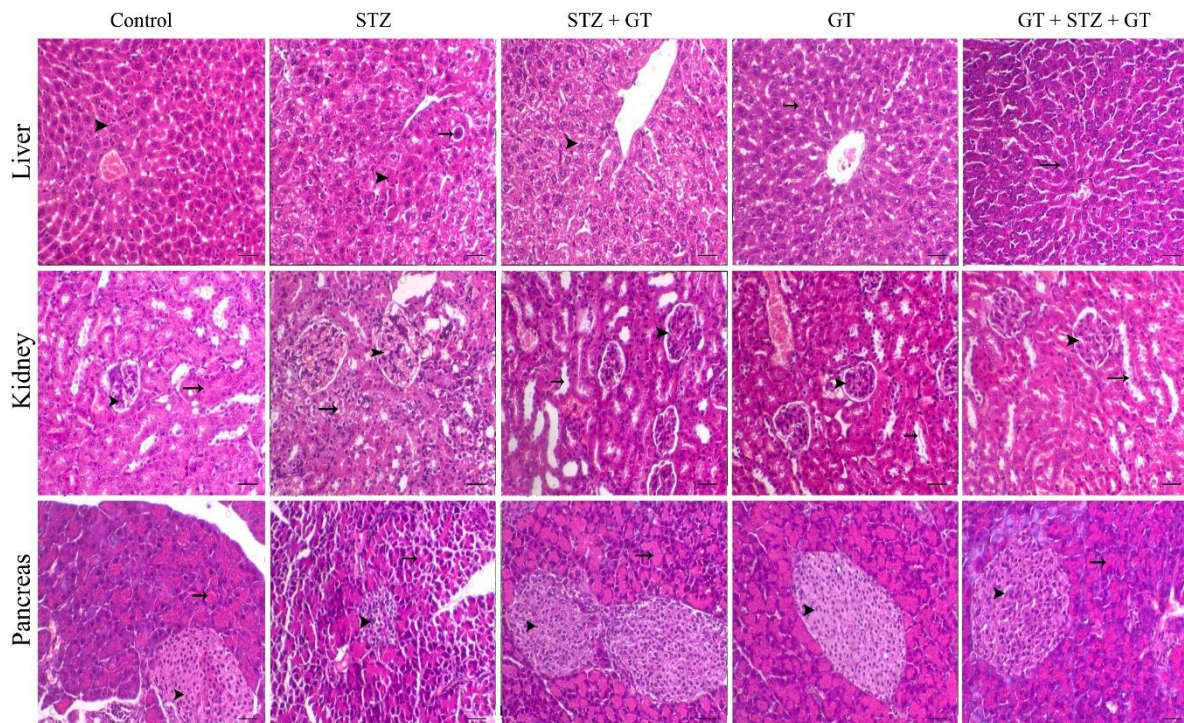


Figure 2: Histomicrograph of liver, kidney and pancreas of control group, STZ (diabetic) group, STZ+GT (green tea-treated STZ) group, GT (green tea) group and GT+STZ+GT (green tea-protective STZ) group. H&E, X200

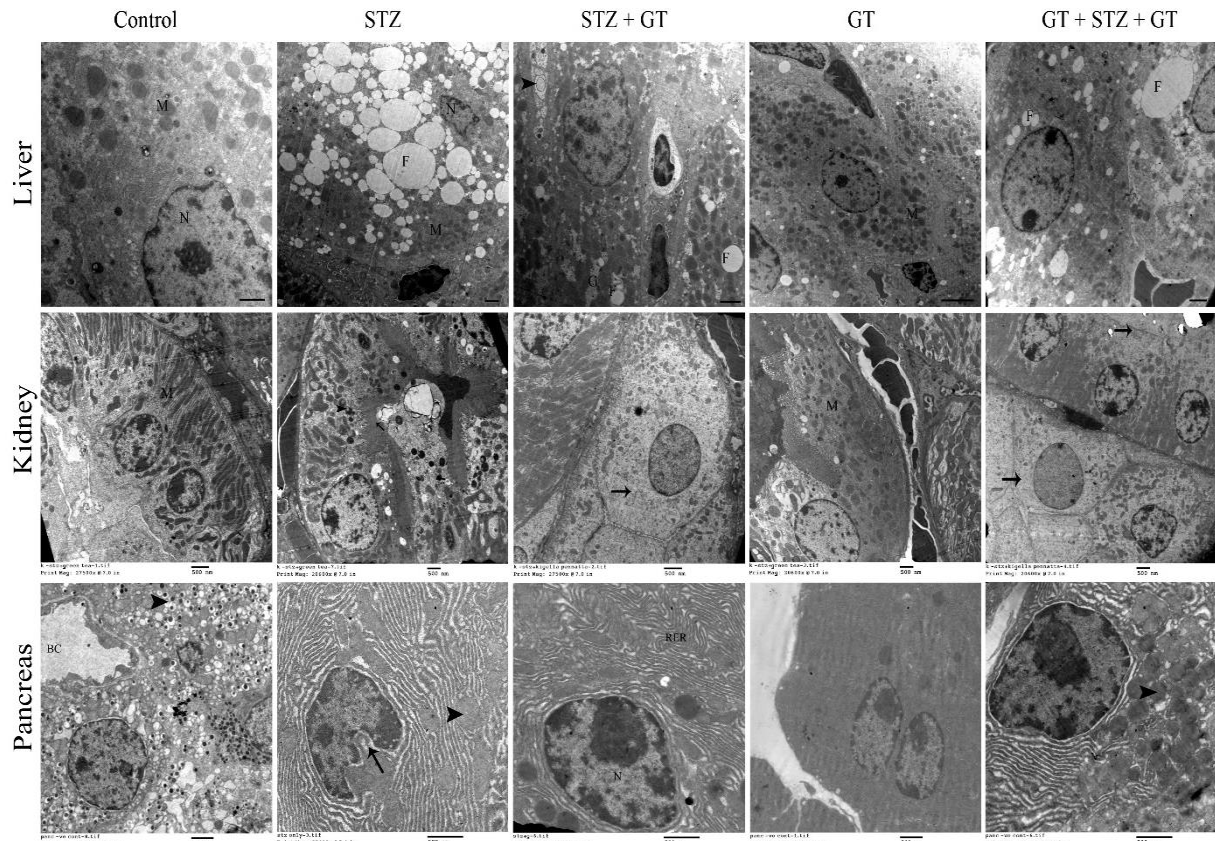


Figure 3: Electron micrograph of liver, kidney and pancreas of the control group, STZ (diabetic) group, STZ+GT (green tea-treated STZ) group, GT (green tea) group and GT+STZ+GT (green tea-protective STZ) group. Scale bar = 500 μ m

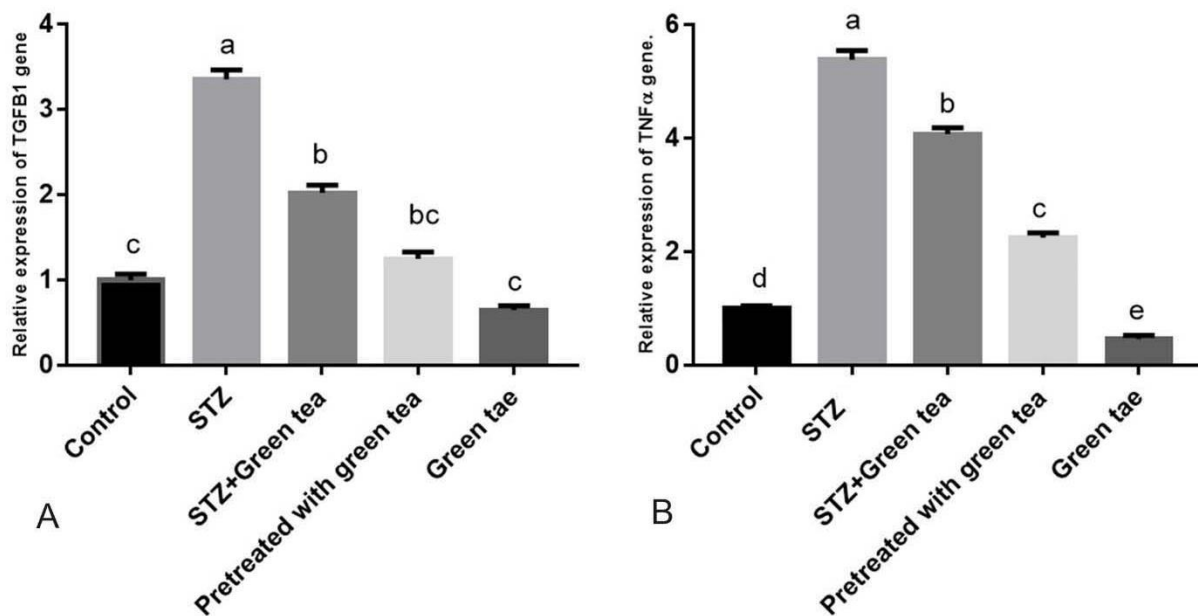


Figure 4: A. Effect of streptozotocin, green tea on the relative expression of *TGFβ1* gene. Means within the same column carrying different superscript letters are significantly different ($P \leq 0.05$). B. Effect of Streptozotocin, green tea on the relative expression of *TNFα* gene. Means within the same column carrying different superscript letters are significantly different ($P \leq 0.05$)

Discussion

Diabetes is a metabolic ailment characterized by hyperglycemia. Its consequences from faults in insulin creation and this leads to disturbance in the metabolism followed by worsening of muscles which leads diabetic complications (36).

Our result revealed that diabetic mice had a significant drop in body weight as shown in figure 1. This result was consistent with (37-39) who reported momentous reduction in body weight in diabetic mice and attributed this effect to the highly muscle wasting and damage of tissue proteins.

Diabetic mice have a marked rise in the levels of serum glucose, triglycerides, cholesterol, LDL-C, VLDL-C when compared with control groups. The obtained data were parallel to that reported by (40) who found that diabetes induces substantial surges in the levels of these parameters and attributed this effect to insulin deficiency that hinder lipoprotein lipase triggering hypertriglyceridemia.

On the other hand, serum levels of insulin, total protein and HDL-C statistically decreased in STZ mice as compared to the control groups and these results agreed with (41-43) who reported similar reduction in these parameters in diabetic animals. Serum total protein in diabetic animals is obviously related to loss of structural proteins and drop in body weight which induced as a result of deficiency of insulin in diabetic mice.

Result revealed that, green tea extract in diabetic mice resulted in noteworthy regaining in body weight, decreases serum glucose in diabetic one, decrease cholesterol, triglyceride, and LDL-C levels, and improved serum total protein and HDL-C. In parallel, (44) also reported that administration of green tea extract in diabetic mice resulted in similar improvement and attributed them to the antihyperglycemic and hypolipidemic activity of green tea (45). Moreover, daily administration of green tea to diabetic mice decreased glucose levels and HbA1c levels and this result was an in consistency with (47).

In the present study, diabetic mice showed a noteworthy rise in MDA relative to control animals. However, pre and post-treatment of diabetic animals with green tea lead to a marked decrease in MDA level. These results was compatible with that obtained by (46) who reported that increase in MDA can be the result of diabetic complications.

MCV, MCH, and MCHC markedly increased in diabetic mice as compared to control mice and suggesting occurrence of anemia in diabetic mice. This result agreed with (50) who reported that anemia in diabetes was due to the increased non-enzymatic glycosylation of RBC membrane proteins, which associated with hyperglycemic oxidation of this glycosylated membrane. In contrast, green tea normalized the elevated MCV, MCH, and MCHC of diabetic mice. These results were consistent with those obtained by (50) who attributed this effect to the decreased lipid peroxide in RBC membrane which decreases hemolysis. The disrupted body defense mechanism of the diabetic animal against infections was distressed due to the neutrophil role in diabetes (48). The obtained results were similar to those obtained by (49) who reported that diabetic mice had a decreased WBC count and attributed this to Streptozotocin ability to suppress the immune system through hindrance of leukocytosis in the bone marrow. We also found a decrease in platelets levels in diabetic mice. In parallel, (51) also reported that platelet accumulation ability in diabetic mice could be due to deficiency of insulin. In contrast, green tea can return this reduced level to a level comparable to that of the control animals. These results are consistent with (52) who reported this effect to green tea ability to increase the biosynthesis of clotting factors. Similar to results obtained by (53), we found no significant change in LH, FSH and estrogen levels among the groups.

Green tea treatment led to a significant down-regulation of *TGFβ1* gene expression in liver. These results agreed with (54) who stated that green tea decreased hepatic expression of *TGFβ1* and this accountable for reduction collagen synthesis and decrease fibrosis. Administration of green tea also led to significant

downregulation of *TNF α* gene expression. In inconsistency, (55) established that green tea persuaded growth inhibition and apoptosis by decrease *TNF α* expression. *TNF α* expression may also have a valuable effect on diabetes since *TNF α* is intricate in developing diabetes.

Histopathological study and transmission electron microscope study revealed that administration of green tea can normalize the liver, kidney, pancreas and spleen that were deteriorated by STZ. In consistency, (56) reported that polyphenols (main ingredients in green tea) have anti-diabetic effects (57).

Conclusion

From the data found in this study, we can conclude that green tea has antidiabetic action though down-regulation of *TGF β 1* and *TNF α* gene expression in liver, improvement of lipid metabolism and body weight in streptozotocin-induced diabetic mice. This study verified the protective action of green tea on experimentally induced diabetic mice.

Conflict of interest

All authors declare that they have no conflict of interest.

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