Antidiabetic and Immunomodulatory Effects of Oleuropein and Vitamin C in Diabetic Male Rats

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Abstract:
The present work was designed to study the antidiabetic and immunomodulatory effects of olive leaf polyphenol (Oleuropein) and vitamin C in diabetic male rats. Diabetes was induced by a single i.p. dose of STZ (40 mg/kg b.w.). Pure oleuropein compound (5 mg/kg b.w.) and vit. C (150 mg/kg b.w.) were orally administered once per a day for 15 days after diabetes induction. Oleuropein and vitamin C showed a significant role in attenuating the blood glucose, insulin, pancreatic amylase, and hexokinase alterations and caused an elevation of antioxidant enzymes in diabetic rats. Oleuropein and vitamin C showed a hematopoetic action as documented by the increase in RBC, Hb, Hct, PLt, decrease in WBC and improved RBC structure. Oleuropein and vitamin C significantly attenuated the oxidative status of diabetic rats. Oleuropein and vitamin C improved the different changes in some cytokines and interleukines and decreases the gene expression of TNF-α, COX2, and caspase3 involved in apoptosis and inflammation. The immunohistochemical sections of pancreas in the diabetic rats treated with oleuropein and vitamin C showed a high intensity of insulin in β-cells. Oleuropein as a natural active compound has antioxidant activity more or less like vitamin C to attenuate the effect of STZ-induced diabetes.

Key words: Oleuropein, Vitamin C, Diabetes, Oxidative Stress, Immunity, Male Rats.

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1. Introduction:

Diabetes mellitus is a global problem characterized by hyperglycemia, progressive β-cell destruction, as a result of chronic insulin resistance and the loss of β-cell mass and function. Insulin deficiency, in turn, leads to chronic hyperglycemia with disturbances in carbohydrate, fat and protein metabolism [1]. In the Middle East and North Africa region, 32.6 million or 9.1% of the population now have diabetes and this number is expected to double in less than 20 years [2].

Hyperglycemia might also promote the generation of reactive oxygen species (ROS) inducing oxidative stress in β-cells. The oxidative stress results in the production and release of pro-inflammatory mediators, which have been known for their involvement in β-cell dysfunction leading to insulin resistance and inflammation attributing to DM [3].

The doubts about the efficacy and safety of the oral hypoglycemic agents have prompted a search for safer and more effective drugs in the treatment of diabetes. There has been a considerable interest in finding natural antioxidants from plant materials to replace synthetic ones [4]. Therefore, it seems that plants, particularly those with high levels and strong antioxidant compounds have an important role in improving the disorders involving oxidative stress such as diabetes mellitus [5].

The olive tree (Olea europaea) is a species of evergreen tree belonging to the family Oleaceae and is native to the coastal areas of the Mediterranean, Asia and Africa. Olea europaea products have been consumed for centuries for health maintenance and treatment of diseases [6]. The fruits as well as the leaves have been recognized for medical purposes because of their phenolic content. The main phenolic compounds are the secoiridoids (Oleuropein, Ole) and flavonoids, these have shown the ability to influence human and animal inflammatory and metabolic biomarkers [7]. Ole mainly present in different parts of the olive such as the flowers, branches, seeds, buds, roots, and oils [8]. Ole has been suggested to have multiple health perspectives such anticancer, anti-arrhythmic, antiviral, spasmolytic, antiviral, cytostatic, hypotensive, antioxidant, and anti-inflammatory [9]. In addition, ole has a cardioprotective effect, anti-ischemic, and hypolipidemic effects [10].

Vitamin C (Vit. C) is a non-enzymatic antioxidant in plasma and tissues [11]. Even in small amounts Vit. C can protect indispensable molecules in the body, such as proteins, lipids, carbohydrates, and nucleic acids from damage by free radicals and reactive oxygen species (ROS) that are generated during normal metabolism, by active immune cells and through exposure to toxins and pollutants. Vit. C concentrations as well as antioxidants have been found to reduce in diabetic patients with respect to healthy controls [12].

Therefore, the present study aimed to investigate the antidiabetic and immunomodulatory effects of olive leaf polyphenol (Oleuropein) and Vit. C in streptozotocin-induced diabetic male rats.

2. Materials and Methods

2.1. Chemicals

STZ and oleuropein were obtained commercially from Sigma-Aldrich Co. Germany. L-ascorbic acid (Vitamin C) was purchased from Oxford Laboratory.

2.2. Experimental Animals

The experiments were permitted on male rats of Wister strain weighing 180±20g. They were obtained from the Department of Physiology, Faculty of Medicine, Alexandria University. The rats were kept in stainless cages for one week before the experimental work and maintained on a standard diet and water available ad libitum. The temperature in the animal room was maintained at 22±2°C with a relative humidity 55±5% and the light was on 12/12 h light/dark cycle. The local committee approved the design of the experiments and the protocols were carried out according to the guidelines of the National Institutes of Health (NIH).
2.3. Experimental Design

The rats were randomly divided into four groups (10 rats each):

**Group 1**: Control group: The rats of this group were intraperitoneally received citrate buffer (pH: 4.5).

**Group 2**: Diabetic group: The rats of this group were intraperitoneally received STZ as a single dose (40 mg/kg b.w.) [13].

**Group 3**: Diabetic+Oleuropein group: The rats of this group were intraperitoneally received STZ (40 mg/kg b.w.) and oleuropein orally (5 mg/kg b.w.) [14].

**Group 4**: Diabetic + Vitamin C group: The rats of this group were intraperitoneally received STZ (40 mg/kg b.w.) and vitamin C orally (150 mg/kg b.w.) [15].

The experiment was continued for 15 days after diabetes induction.

2.4. Induction of diabetes

The rats were made to fast overnight before the induction of diabetes by a single intraperitoneal injection of 40 mg/kg b.w. STZ freshly prepared with citrate buffer (pH 4.5). Hyperglycemia was confirmed 3 days after injection by measuring the tail vein blood glucose level with an Accu-Check Sensor Comfort glucometer. Only the animals with fasting blood glucose levels >200 mg/dl were selected for this study [16].

2.5. Collection of blood and serum

At the end of the experiment, the rats were fasted overnight and sacrificed after light chloroform anesthesia. The first part of blood was collected into heparinized tubes for the determination of hematological parameters (RBCs, Hb, Hct, WBCs, and platelet counts). The remaining non-heparinized blood was allowed to clot in a centrifuge tube and the sera were isolated at 3000 rpm for 15 min at 4°C. The sera were collected in clean tubes and stored at -20°C until required.

2.6. Determination of hematological parameters

The hematological parameters, including RBCs, Hb, Hct, WBCs and PLt were estimated by Particle Counter (ERMA Inc., Tokyo. Model PCE-210).

2.7. Preparation of pancreas tissue homogenates

About 500 mg of tissue was homogenized in 2 ml phosphate buffered saline (w/v: 500 mg tissue with 4 ml PBS, pH: 7.4). Homogenates were centrifuged at 10.000 rpm for 15 min at 4°C. The supernatant was collected in clean tubes and stored at -20°C for determination of antioxidant enzyme activities and lipid peroxidation markers.

2.8. Biochemical parameters

Glucose level was measured as reported by Braham and Trinder [17] method. Glycosylated hemoglobin (HbA1c) was estimated by fast ion-exchange resin separation method [18]. Insulin, pancreatic amylase, and hexokinase were determined according to the methods of Finlay and Dillard [19], Pulse and Schmidt [20] and Gubern et al. [21], respectively. Pancreas TBARS and GSH were measured according to Tappel & Zalkin [22] and Richardson and Mwephy [23] methods, respectively. Also, the antioxidant enzyme activities, including superoxide dismutase (SOD) [24], catalase (CAT) [25], glutathione-S-transferase (GST) [26], and glutathione peroxidase (GPx) [27] were assayed in pancreas homogenates.

2.9. Determination of cytokines and interleukines
Quantitative measurements of rat IL-1β, IL-2 and IL-6 according to the methods of Chan & Perlstein [28], Ansar et al. [29], and Ferguson-Smith et al. [30] were estimated. CD4+ and CD8+ were determined by using the method of Aboulker et al. [31]. IgG (Kamiya Biomedical Company Cat. No. KT-418) and IgM (Abcam ab-157738) were determined according to the manufacturer’s instructions of kits.

2.10. Molecular studies:

2.10.1. Isolation of the total RNA:

Total RNA was extracted from the pancreas tissues according to Chomczynski & Sacchi [32]. The procedure using GStractTM RNA Isolation Kit II Guanidinium Thiocyanate Method.

2.10.2. Determination of RNA concentration and purity.

RNA concentration was determined by measuring the absorbance at 260 nm (the RNA solution was diluted 5/495 μl with RNAase free water). The concentration was calculated using the following equation: 1 absorbance unit at 260 nm corresponds to approximate concentration of 40 μg/ml of single-stranded RNA. Quality of RNA preparations was confirmed by calculating 260/280 ratio for detection of protein contamination and by running samples on agarose to confirm that the samples are DNA-free.

2.10.3. Reverse -transcriptase poly chain reaction (RT-PCR).

Alteration in the steady state mRNA levels of genes relevant to oxidative stress in different groups of experimental rats is determined using reverse-transcriptase PCR analysis. Using one-step RT-PCR (RT/PCR Master Mix Gold Beads, BIORON) reaction, the cDNA was synthesized and used for amplification of the target gene (s).

2.10.4. Quantitative RT-PCR assay (qRT-PCR)

Quantitative RT-PCR was used to measure the mRNA expression levels of TNF-α and COX2 genes with β-actin gene as a housekeeping gene. cDNA was synthesized by High-Capacity cDNA Reverse Transcription Kit according to the manufacture protocol. The fold difference was calculated by the formula $2^{\Delta\Delta C_t}$ [33] (Table 1).

**Table (1): The primer sequences and product size of target genes in expected PCR products for RT-PCR.**

| Primers sequence and condition | Product size (bp) | Reference |
|-------------------------------|------------------|-----------|
| -β-actin                      | 565bp            | [34]      |
| F: 5’-GGCATCCTGACCTGAAGTA-3’  |                  |           |
| R: 5’-GCC GAT AGT GAT GAC CTG ACC-3’ |      |           |
| 94°C for 45s, 60°C for 45s, 72°C for 45s |  |           |
| Number of cycles: 35          |                  |           |
| - TNF-α                       | 320bp            | [35]      |
| F: 5’CTCTTCTCCTCCTTGATCGTGGA3’ |                  |           |
| R: 5’GAAAGCATGATCCGGGACGTAAG3’|                  |           |
| 94°C for 30sec, 53°C for 30sec, 72°C for 1 min |  |           |
| Number of cycles: 35          |                  |           |
| -COX-2                        | 162bp            | [34]      |
| F: 5’TGTATGCTACATCTGGCTTCGG   |                  |           |
| R: 5’GTTTGAAACGAGTCTCGTCATC   |                  |           |
| 94°C for 30sec, 56°C for 30sec, 72°C for 1 min |  |           |
| Number of cycles: 35          |                  |           |
2.11. Sample preparation and viewing–SEM

Fresh specimens of blood were immediately taken and fixed in 0.1 ml/l phosphate buffer (pH: 7.4) containing 2.5% glutaraldehyde and 2% paraformaldehyde at 4°C for 3 hours. Blood specimens were then fixed in 2% osmium tetroxide (OSO$_4$) in the same buffer at 4°C for 2 hours. Samples were washed in phosphate buffer and dehydrated in a graded series of ethanol. The samples were dried by means of the critical point method mounted, using carbon paste on an Al-stub and coated with gold up to a thickness of 400Å in a sputter-coating unit (JFC-1100 E). The processed specimens were examined and photographed by Jeol-JSM 5300 scanning electron microscope, operated between 15 and 20 kV according to Moustafa and Basra [36].

2.12. Determination of insulin intensity by immunohistochemical procedure in pancreas

The pancreatic tissues were fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned at 5 ml thickness. Immunocytochemical reaction was performed according to the Avidine Biotine technique described by Hsu et al. [37]. The intensity of insulin in β-cells was calculated by quantification of immunohistochemistry staining (The anti-insulin antibody) using Image J software.

2.13. Statistical analysis

All statistical analyses were conducted by using the statistical package SPSS version 22.0 (Chicago, IL). Values were compared by one-way analysis of variance (ANOVA). Post-hoc testing was performed for inter-group comparisons using the least significant difference (LSD) test at $p \leq 0.05$.

3. Results:

3.1. The effects of Ole and Vit. C on glucose, insulin, HbA1c, α-amylase and hexokinase of male rats.

Table 2 showed that STZ caused significant ($P \leq 0.05$) increase in the glucose and HbA1c while, insulin, α-amylase and hexokinase were significantly ($P \leq 0.05$) decreased. As a result of Ole and Vit. C treatments the levels of glucose, insulin, HbA1c, hexokinase and α-amylase were improved in the diabetic rats. It was obvious that the Ole has the most curative effect against increment in glucose, HbA1c and α-amylase and reduction in insulin.

**Table 2: The effects of Ole and Vit. C on glucose, insulin, HbA1c, α-amylase and hexokinase of male rats.**

| Parameter               | Experimental groups |
|-------------------------|---------------------|
|                         | Control            | Diabetic          | Diabetic+Ole    | Diabetic+Vit. C |
| Glucose (mg/dl)         | 96.43±2.53         | 324.37±86.94$^a$ | 109.73±5.09$^{bc}$ | 145.20±17.38$^b$ |
| Insulin (μIU/ml)        | 23.06±0.33         | 7.04±0.12$^a$    | 18.79±0.39$^{bc}$ | 10.08±0.56$^{ab}$ |
| HbA1c (%)               | 4.35±0.09          | 16.32±0.95$^a$   | 6.11±0.22$^{bc}$  | 6.68±0.31$^{ab}$  |
| α-amylase (IU/L)        | 173.83±9.13        | 161.21±8.50$^{a}$| 169.50±3.27$^{ab}$ | 160.87±7.82$^{bc}$ |
| Hexokinase (μU/mg protein) | 127.83±0.96       | 86.15±0.87$^a$   | 107.62±1.49$^{abc}$ | 95.08±1.22$^{ab}$ |
The values are expressed as mean±SE.

a. The mean values are significantly different in comparison with control group (P≤0.05).

b. The mean values are significantly different in comparison with diabetic group (P≤0.05).

c. The mean values are significantly different in comparison with diabetic +Ole group and diabetic +Vit. C (P≤0.05).

3.2. The effects of Ole and Vit.C on the hematological parameters of male rats.

Results pointed in table 3 showed that STZ caused significant (P≤0.05) decrease in RBC, Hb, Hct, and PLt. On the other hand, WBC, LY and GR were significantly (P≤0.05) increased in untreated diabetic rats. However, diabetic rats treated with Ole and Vit. C exhibited significant (P≤0.05) increase in RBC, Hb, Hct, and PLt and decrease in WBC, LY, MO and GR.

Table 3: The effects of Ole and Vit. C on the hematological parameters of male rats.

| Parameter     | Experimental groups |
|---------------|---------------------|
|               | Control             | Diabetic            | Diabetic+Ole | Diabetic+Vit. C |
| RBCs (×10⁶/µl) | 6.55±0.28           | 5.71±0.08ab         | 6.00±0.24b   | 6.04±0.90b      |
| Hb (g/dl)     | 14.86±0.48          | 12.22±0.11a         | 12.38±0.04ab | 12.43±0.13ab    |
| Hct (%)       | 32.50±0.36          | 27.93±0.43a         | 31.02±0.54ab | 31.30±0.34abc   |
| PLt (×10³/µl) | 633.67±24.87        | 412.40±12.55a       | 589.60±27.27b| 621.80±21.65bc  |
| WBC (10³ Cell / µL) | 3.23±0.76          | 4.94±0.22a          | 3.83±0.84abc | 4.57±0.92a      |
| LY (%)        | 87.56±0.69          | 47.81±1.35a         | 84.00±3.28bc | 83.00±3.21b     |
| MO (%)        | 11.94±0.28          | 19.37±0.19a         | 14.00±0.16abc| 16.00±0.64ab    |
| GR (%)        | 1.90±0.12           | 32.70±1.35a         | 2.00±0.22abc | 1.00±0.81ab     |

* The values are expressed as mean±SE.

a. The mean values are significantly different in comparison with control group (P≤0.05).

b. The mean values are significantly different in comparison with diabetic group (P≤0.05).

c. The mean values are significantly different in comparison with diabetic+Ole group and diabetic+Vit. C (P≤0.05).

3.3. The effects of Ole and Vit. C on oxidative stress markers in the pancreas of male rats.

Concerning with the lipid peroxidation markers, table 5 showed a significant (P≤0.05) increase in TBARS of untreated diabetic group, however, the GSH was significantly decreased as compared to the control group. On the other hand, treatment with Ole and Vit. C caused a significant (P≤0.05) decrease in TBARS and an increase in GSH compared to untreated diabetic group.
Table 5: The effects of Ole and Vit. C on oxidative stress markers in the pancreas of male rats.

| Parameters         | Control     | Diabetic    | Diabetic+Ole | Diabetic+Vit. C |
|--------------------|-------------|-------------|--------------|----------------|
| TBARS (nmol/g tissue) | 12.91±1.24  | 32.47±1.32  | 14.64±0.69abc | 18.64±0.97a    |
| GSH (µmol/g tissue)  | 32.97±1.14  | 18.49±0.72a | 26.41±0.76abc | 24.34±1.37a    |

* The values are expressed as mean ± SE.

a. The mean values are significantly different in comparison with control group (P≤.0.05).

b. The mean values are significantly different in comparison with diabetic group (P≤.0.05).

c. The mean values are significantly different in comparison with diabetic +Ole group and diabetic +Vit. C (P≤.0.05).

3.4. The effects of Ole and Vit. C on the antioxidant enzyme activities in the pancreas of male rats.

According to the present data, it was clearly showed that SOD, CAT, GPx and GST were significantly (P≤0.05) decreased in the diabetic group as compared to the control group (Table 4). Diabetic rats treated with Ole and Vit. C exhibited significant (P≤0.05) increase in the antioxidant enzyme activities as compared to untreated diabetic rat. It was evident that Ole has an antioxidant effect more than Vit. C.

Table 4: The effects of Ole and Vit. C on the antioxidant enzyme activities in the pancreas of male rats.

| Parameters         | Control     | Diabetic    | Diabetic+Ole | Diabetic+Vit. C |
|--------------------|-------------|-------------|--------------|----------------|
| SOD (U/mg protein) | 1.20±0.02   | 0.98±0.01abc | 1.09±0.01abc | 0.93±0.01a     |
| CAT (U/mg protein) | 58.60±10.52 | 40.89±0.17abc | 46.51±0.59abc | 42.81±0.21a    |
| GPx (U/mg protein) | 1.23±0.05   | 0.88±0.05a  | 1.15±0.09abc | 0.89±0.03a     |
| GST (µmol/mg protein) | 5.85±0.12 | 4.55±0.03a  | 4.80±0.05abc | 4.69±0.07a     |

* The values are expressed as mean ± SE.

a. The mean values are significantly different in comparison with control group (P≤.0.05).

b. The mean values are significantly different in comparison with diabetic group (P≤.0.05).

c. The mean values are significantly different in comparison with diabetic +Ole group and diabetic +Vit. C (P≤.0.05).

3.5. The effects of Ole and Vit. C on IL-1β, IL-6, IL-2, CD4+, CD8+, IgG and IgM of male rats.

IL-1β, IL-2, IL-6, CD8+, CD4+, and IgG were significantly (P≤0.05) increased in the diabetic group compared to the control group. While, IgM was significantly decreased (Table 6). Ole and Vit. C treatment caused significant (P≤0.05) decrease in the IL-1β, IL-2, IL-6, CD8+, CD4+, IgG and significant (P≤0.05) increase in the IgM compared to the untreated diabetic group. It was obvious that the Ole has the most curative effect against changes in interleukins and cytokines.
Table 6: The effects of Ole and Vit. C on IL-1β, IL-6, IL-2, CD4⁺, CD8⁺, IgG and IgM of male rats.

| Parameter | Control | Diabetic | Diabetic+Ole | Diabetic+Vit. C |
|-----------|---------|----------|--------------|----------------|
| IL-1β (pg/ml) | 107.86±3.42 | 169.86±2.70⁰ | 130.14±3.12ab,c | 140.29±2.79ab |
| IL-6 (pg/ml) | 418.00±2.38 | 467.86±3.45⁰ | 436.86±3.03ab,c | 458.71±1.86ab |
| IL-2 (pg/ml) | 10.29±0.57 | 24.71±0.97⁰ | 15.43±0.48ab,c | 21.00±0.44ab |
| CD4⁺ (%) | 32.63±0.80 | 41.94±0.62⁰ | 36.24±0.85ab,c | 38.57±0.44ab |
| CD8⁺ (%) | 30.91±0.93 | 40.30±0.61⁰ | 34.67±1.11ab,c | 40.36±0.44a |
| IgG (mg/mL) | 4.38±0.06 | 6.70±0.06⁰ | 5.17±0.04ab,c | 5.57±0.15ab |
| IgM (mg/mL) | 0.46±0.007 | 0.38±0.005⁰ | 0.41±0.003ab | 0.40±0.007ab |

* The values are expressed as mean ± SE.
  a. The mean values are significantly different in comparison with control group (P≤0.05).
  b. The mean values are significantly different in comparison with diabetic group (P≤0.05).
  c. The mean values are significantly different in comparison with diabetic +Ole group and diabetic +Vit. C (P≤0.05).

3.6. The effects of Ole and Vit. C on TNF-α and COX-2 in the pancreas of male rats.

Table 7 showed that TNF-α and COX-2 were significantly (P≤0.05) increased in the diabetic group compared to the control ones. Treatment of diabetic rats with Ole and Vit. C caused significant (P≤0.05) decrease in the TNF-α and COX-2 compared to untreated diabetic group.

Table 7: The effects of Ole and Vit. C on TNF-α and COX-2 (Fold changes calculated in reference to the control group) in the pancreas of male rats.

| Parameter | Control | Diabetic | Diabetic+Ole | Diabetic+Vit. C |
|-----------|---------|----------|--------------|----------------|
| TNF-α     | 1.00±0.00 | 4.39±0.00⁰ | 1.84±0.00⁰b,c | 2.47±0.00⁰a,b |
| COX-2     | 1.00±0.00 | 2.17±0.00⁰ | 1.34±0.00⁰b,c | 1.70±0.00⁰a,b |

* The values are expressed as mean ± SD.
  a. The mean values are significantly different in comparison with control group (P≤0.05).
  b. The mean values are significantly different in comparison with diabetic group (P≤0.05).
  c. The mean values are significantly different in comparison with diabetic +Ole group and diabetic +Vit. C (P≤0.05).

3.7. The effects of Ole and Vit. C on the diameter of red blood cells in male rats.

Table 8 showed that diabetic group exhibited significant (P≤0.05) decrease in the diameter of red blood cells compared to the control group. On the other hand, treatment of diabetic rats with Ole and Vit. C improved these alterations in the RBC diameter.
Table 8: The effects of Ole and Vit. C on the red blood cells diameter in male rats

| Parameter | Control                     | Diabetic                  | Diabetic+Ole | Diabetic+Vit. C |
|-----------|-----------------------------|---------------------------|--------------|----------------|
| Diameter of red blood cells (μm) | 2.44±0.06                  | 1.59±0.15<sup>a</sup>   | 2.41±0.04<sup>ab</sup> | 2.43±0.03<sup>ab</sup> |

* The values are expressed as mean ± SE.

<sup>a</sup> The mean values are significantly different in comparison with control group (P≤.0.05).

<sup>b</sup> The mean values are significantly different in comparison with diabetic group (P≤.0.05).

<sup>c</sup> The mean values are significantly different in comparison with diabetic +OLE group and diabetic +Vit. C (P≤.0.05).

3.8. The effects of Ole and Vit. C on the morphological pattern of RBC:

As shown in Fig. 1A normal erythrocytes are concentric type double concave discs, with smooth surface, neat margin, uniform dispersion, and abnormal forms are rare. Diabetic group shows different morphological alterations in RBC including, stomatocyte cells, target cells, echinocyte cells and triconcave cells, and tear drop (B1, B2 &B3). Treatment of diabetic rats with Ole and Vit. C improved these alterations in the RBC morphology (C&D). It was observed that Vit. C has the most curative effect against the RBC morphological alterations.
Figure 1: SEM micrographs of RBC Control group (A) showing normal typical morphology of RBC, diabetic group (B1, B2&B3) showing RBCs abnormalities; stomatocyte cells (yellow arrow), target cells (blue dotted arrow), echinocyte cells and triconcave cells (green arrow & red dotted arrow) and tear drop (purple arrow), diabetic+oleuropein-treated group and diabetic +Vitamin C-treated group showing slightly normal RBCs morphology with relatively few alterations.

3.9. The effects of Ole and Vit. C on the insulin expression in β-cell

The pancreatic tissues of control rat showed a strong positive insulin expression in β-cells of Langerhans with well-defined islets (Figure 2A). The pancreatic tissues of diabetic rats showed a weak insulin expression in β cells of Langerhans with few insulin immune-stain cells (Figure 2B1&2B2). On the other hand, the pancreatic tissues of diabetic rats treated with Ole and Vit. C (Diabetic+Ole & diabetic+Vit. C-treated rats) showed a positive insulin expression in β cells of Langerhans (Figure 2C&2D).
**Figure 2:** A photomicrograph of the pancreatic tissues of control rat showing: strong positive insulin expression in β-cells with well-defined islets of Langerhans (black arrow) (Immune stain, X 400). **B1&B2** photomicrographs of the pancreatic tissues of diabetic rat (STZ-treated rat) showing: weak insulin expression in β cells of Langerhans with few insulin immune-stain cells (Yellow arrow) (immune stain, X 400). **C&D** photomicrographs of the pancreatic tissues of diabetic rats treated with Ole and Vit. C, respectively (STZ+Ole&STZ+Vit.C treated rats) showing: increase insulin expression in β cells of Langerhans (Black arrow immune stain, X 400).

4. **Discussion**

Streptozotocin (STZ) acts as a diabetogenic because it inhibits production of insulin and selectively destroys the insulin-producing β-cells by inducing necrosis as reported by Busineni et al. [38]. The present results were in the same line with Yang and Kang [39]; Mohamed and Abdou [40] who stated that the glucose level steadily increased in the STZ-treated group compared with normal control group. Also, diabetes mellitus is known to result in significant decrease in α-amylase and hexokinase levels. Mufeed et al. [41] indicated that the low α-amylase activity in diabetics correlated negatively with hyperglycemia and duration of diabetes. The hepatic hexokinase activity of diabetic rats is almost entirely inhibited or inactivated due to the lack of insulin [42].

Oleuropein (Ole) has hypoglycemic effect that induced insulin release and act as a gluicosidase inhibitor, reducing the absorption of carbohydrates in the gut, resulting in a reduction of plasma glucose concentration [43]. Fujiwara et al. [44] suggested the possibility of Ole to be effective for type 2 diabetes by reducing insulin resistance in skeletal muscles. Ole is associated with improved glucose metabolism in diabetic rats [45] and healthy subjects [46].
Vit. C plays a chief role in ameliorating insulin resistance of diabetic patients due to its antioxidant function [47]. The consumption of foods high in ascorbic acid has been associated with lower risk of diabetes [48]. Vit. C supplementation reduces blood glucose level and improves glycosylated hemoglobin in type 2 diabetes as reported by Ashor et al. [49].

The diabetic rats exhibited an anemic state as documented by reduction in RBC, Hb, Hct. These results consistent with Ajiboye et al. [50]. Hyperglycaemia leads to reduced deformability, changes in mechanical properties of RBCs, increased adhesion and osmotic fragility, leading to changes in erythrocyte structure and hemodynamic characteristics as reported by Khalafallah et al. [51]. Also, hyperglycemia causes an increase in the production of lipid peroxides, a marker of oxidative stress in diabetes, which consequently have toxic effects that lead to hemolysis of red blood cell [52]. The DM is accompanied by the development of hypoxia that disturbs the physiochemical properties of the erythrocyte membrane that leads to anemia [53]. The intraperitoneal injection of STZ to rats significantly reduced the WBC count and its differential. The reduction in these parameters could be linked to suppress leucocytosis from the bone marrow which may account for poor defensive mechanisms against infection. Direct toxicity of STZ on lymphocytes, particularly on CD8+ cells and B cells was shown in vitro [54].

Palanisamy and Mariamichael [55] stated that the morphological features of RBCs exhibited a statistically significant difference (P <0.01) between the normal and diabetic cells, suggesting that it could be helpful in the diagnosis of diabetes mellitus. Neamtu et al. [56] observed a high prevalence of the red cell morphology changes in diabetic patients compared with non-diabetic subjects. They recorded different changes in RBCs including, anulocytes type, red cells in "mark to the target fired" (codocytes), bream (leptocytes), schizocytes, and red cells in "drop" (dacrucytes).

A curative effect of Ole against the anemia and the morphological alterations in RBCs of diabetic rats may be due to its ability to scavenge and trap free radicals. These results may be due to Ole which acts as a flavonoid that could restore membrane protein aggregation caused by oxidative damage to some extent [57]. A significant protective effect on human blood leucocytes by Ole was observed [58]. It is suggested that the antioxidant effect of Ole may be able to reduce leukocyte lipoxygenase enzymes and inhibiting their ability to release ROS whilst stimulates the generation of prostaglandins, which act as immune-modulator. Geyikoglu et al. [59] reported that Ole administration dose dependently increases leucocyte and thrombocyte counts (50 and 100 mg/kg).

Vit. C administration has been shown to decrease hemolysis and reduce oxidative damage to RBC by stabilizing the membrane integrity and decreasing the membrane susceptibility to lipid peroxidation [60]. Vit. C deficiency was associated with anemia, characterized by a decrease in the hemoglobin, reduction in the number of erythrocytes, and hematocrit [61]. In essence, diabetes pathophysiology might produce ascorbate deficiency in RBCs, with potential vascular pathophysiologic consequences. The exciting therapeutic aspect is that local deficiency might be reversible with oral ascorbic acid [62]. Vit. C has been shown to stimulate both the production and function of leukocytes, especially, neutrophils, lymphocytes, and phagocytes [63].

Glycated hemoglobin is a very reliable index to monitor glucose lowering therapy and also for long-term blood sugar control. In persistent hyperglycemia, there is a raise in nonenzymatic glycation, which is formed between glucose and the N-end of the beta chain of Hb, forming glycated hemoglobin. Further, glucose and dicarboxyl compounds can also react with hemoglobin, forming advanced glycation end products, which can contribute to the additional development of diabetes complications [64]. Xiao and Högger [65] stated that Ole similar to others antioxidants such as vitamin E and coenzyme Q10 could reduce HbA1c and prevent hyperglycemia. Oral supplementation of Vit. C reduces FBG, two hours PPBG, and improves HbA1c. Hence, its combination with diabetic drugs may be beneficial in the treatment of T2DM to maintain good glycemic control [66].

Diabetic rats exhibited oxidative status as documented by an increase in the pancreas TBARS. Sagoo and Gnudi [67] showed that STZ induced diabetes in young rats caused a significant increase in the formation of
MDA, NO and a significant reduction in the antioxidant enzymes (SOD, CAT) in the testis. The evidence from both the experimental and clinical studies indicates that oxidative stress plays a major role in the diabetic pathophysiology [68]. The activity of the antioxidant enzymes in the alloxan-induced diabetic rabbits decreased when compared with the non-diabetic rabbits [69].

In this study, Ole antioxidant role may be attributed to its ability to decompose ROS species and trapping radicals before reaching cell membranes [8]. Abo Ghanema and Sadek [70] stated that olive leaf extract improved the erythrocyte enzymatic antioxidant activities such as GPx, SOD and CAT probably due to its rich content from oleuropein. As Ole has two hydroxyl groups which are believed to play a critical role in quenching reactive oxygen species. Ole administered to rats prior to stress attenuated the inhibition of SOD and CAT activities and, thus, additionally implicated its role in the modulation of the oxidative balance in liver [71].

Vitamin C is acting to neutralize free radical damage to cells, including DNA, lipids and proteins. As a free radical neutralizing agent, Vit. C readily donates electrons to unstable molecules and breaks the chain of free radical damage to cells and tissues. Vit. C is an enzymatic co-factor and antioxidant that is capable of shifting between its oxidized and reduced forms by electron donation. By this mechanistic action, it protects the intracellular membrane, DNA, proteins, and lipids against oxidative stress [72].

Many researchers suggested that TNF-α, IL-1, IFN-γ, IL-6, and IL-1β are among the inflammatory cytokines that affect the development of diabetes [73]. In T2DM patients, proinflammatory cytokines playing an important role in the autoimmune pathogenesis of β-cell destruction [74]. Moganti et al. [75] stated that the production and secretion of cytokines (TNF and IL-1β) boost intracellular inflammatory signalling and insulin resistance. Insulin resistance leads to a perturbation in the lipid homeostasis, cytokines, and adipokines production, resulting in increased systemic inflammation, with higher levels of inflammatory markers such as CRP, TNF-α and IL-6 [76].

Olive-derived phenolic compounds, including Ole, can decrease the production of monocyctic inflammatory mediators, decreasing the production of IL-1β in human whole blood cultures stimulated with monocytes-triggered by LPS. The administration of Ole in a mouse model causes a significant reduction in TNF-α, IL-1β and NO [77]. Ole attenuates the inflammatory process in DSS-induced colitis by down regulating the expression of COX-2 and pro-inflammatory cytokines, such as TNF-alpha, IL-1β, IL-6 and IL-17 [78]. Larussa et al. [79] demonstrated that Ole exerts broad anti-inflammatory actions in inflamed colonic tissue from ulcerative colitis (UC) patients.

Supplementation of vitamin C enhances the function of the immune system in adequately nourished individuals [60]. It was suggested that Vit. C may play a significant role in the regulation of the inflammatory response [80]. Vit. C has antioxidative and anti-inflammatory functions, such as scavenging ROS and RNS, preventing the initiation of chain reactions that lead to protein glycation and protecting against lipid peroxidation [81].

In respect to immunohistochemical sections, the present results show destroyed β-cells as documented by low intensity of insulin. These results agree with Qadir et al. [82]. On contrast, Ole and Vit. C supplementation can induce significant improvement in the function of β-cells, as evidenced by the elevated serum insulin level. It has been revealed that regeneration of β-cells and reactivation of insulin secretion after treating diabetic rats with phenolic compound could be due to the possibility that some islet β-cells are able to be reactivated by phenolic compound to labour its insulin secretion [83]. Ascorbic acid supplementation significantly increased islet size, along with the up-regulation of genes that encode proteins involved in β-cell proliferation and differentiation, suggesting a positive role of antioxidant in the proliferation of β-cells in the progression of diabetes [84]. Furthermore, Vit. C intake can ameliorate oxidative stress and insulin resistance [85].
Conclusion

Ole and vitamin C treatment showed an antioxidant and anti-inflammatory effects in diabetes by decreasing oxidative stress and regenerated β-cells function.

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