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Targeting tumor necrosis factor alpha (TNF-α) in diabetic rats could approve avenues for an efficient strategy for diabetic therapy

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ABSTRACT

Background: Several studies held belief that downregulation of TNF-α may be effective for preventing diabetes and its complications. However, it is not known whether TNF-α downregulation in long-term can generate any biological adverse.

Aim: The aim of the present study was to clarify what the impact is for such treatment with specific antibody for TNF-α on the other biological activities after 4 weeks.

Methods: Using western blot, IHC, Elisa, biochemical assays and scanning electron microscope.

Results: Results show that TNF-α, FOXO-1, IL-6 and MPO, when expressed in diabetic rats, collectively induce dramatic changes in diabetic rats. Since, TNF-α is involved in activation of transcription factor FOXO1 along with oxidative stress mediated by neutrophils. On one hand, IL-6 mediates neutrophils activation leading to an augmentation in stress mediators. And FOXO1 is activated in order to eliminate these oxidative mediators, on the other hand. Data show also that the prominent defect in mucosal IgA and IL-2 secrete may be the leading reasons for digestive atrophy. Finally, Akt-1 inhibits the cleavage of caspase 3, so, it could prevent the incidence of apoptosis.

Conclusion: Findings of this study reveal how TNF-α can be mechanistically coupled to greater diabetic complications potential.

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1. Introduction

Diabetes type 1 is a multi-factorial disease characterized by an aggressive destruction of the insulin producing β-cells often mediated by multiple autoimmune reactions. Approximately 440,000 cases of type 1 diabetes in childhood, more than 25% were in south-east Asian region and more than 20% from the European region (International Diabetes Federation) [1]. Diabetes type 2 is a metabolic disorder that is primarily characterized by insulin resistance accompanied by insulin secretion depression that in turn leads to hyperglycaemia. Therefore, the primary repercussion of diabetes is rise in blood glucose levels due to the imperilment in the cellular glucose consumption/uptake. The low insulin levels inhibit the transport of glucose across cell membranes causing a high blood glucose level, which subsequently leads to hyperglycaemia. The complications of diabetes are retinal damage, renal failure and elevation of ketone bodies. In this regard, reactive oxygen species (ROS) could involve in insulin resistance [2]. The streptozotocin, a glucosamine-nitrosourea compound obtained from Streptomyces has been extensively used as an experimental tool to develop animal models to study diabetes and associated complications. Since, it enters pancreatic β cells through glucose transporter 2 channels in the plasma membrane and causes cellular toxicity and local immune responses that lead to hypoinsulinemia and hyperglycemia in animals [3]. Examination of bones from STZ-induced diabetic mice was found to exhibit a significant decrease in bone volume fraction [4] low bone formation. The effect of diabetes on bone stiffness is less clear, with some reports of increased stiffness. Under normal physiological conditions, bone undergoes dynamic microstructural remodeling throughout life to accommodate mechanical stress and calcium demand [5]. Bone remodeling is a coupled process of bone resorption and formation, and requires coordination of all three types of bone cells, namely osteocytes, osteoblasts and osteoclasts [6]. Under toxic insults and or mechanical stress, osteocytes act as mechanosensors to detect changes in the flow of bone fluid within bone canaliculi, and respond by transmitting signals to the osteoblasts via their syncytial processes [6]. Numerous experimental, epidemiological, and clinical studies have established that the proinflammatory cytokines are key mediators in the process of osteoclast differentiation and bone resorption [7–10]. These changes in bone have also established in chronic inflammations due to increased production of proinflammatory cytokines including TNF-α, IL-1, and IL-6. It has also been established that
upregulated proinflammatory cytokines are primary mediators of osteopenia or osteoporosis [11]. Since, the removal of cartilage coupled with bone formation and remodeling is dependent on the coordinated expression of cytokines that initiate and regulate the fracture healing process normal fracture repair process [12].

2. Materials and methods

2.1. Animals and experimental design

Thirty male albino rats weighing approximately 100–120 g were used in this study. The experimental procedures were approved by the Committee of Animal Care and Use at Damanhour University. Rats were arranged into three main groups; 10 rats serve as control group and 20 rats were involved as experimental diabetic groups. Diabetes was induced by intraperitoneal injection of a single dose of streptozotocin (60 mg/kg/BW in citrate buffer pH 4.6). Rats were considered to be diabetic when serum glucose levels exceeded 250 mg/dl (Diagnostics, Indianapolis, IN, USA). A group of diabetic rats received an i.p. injection of anti-TNF-α (10 mg/kg/BW) in 1 ml of phosphate-buffered saline on days 21 and 25. After one month of the last injection, both control and experimental animals were sacrificed and blood, duodenum and femur bones were collected and frozen.

2.2. Biochemical analysis

Plasma cytokines, IL-2, IL-4, IL-6 and TNF-α levels were determined by ELISA using the commercial kits according to the manufacturer’s instructions, as well as, Caspase-3 in duodenum tissue. Mucosal IgA, total proteins and plasma glucose level were determined by commercial kits. For determination of neutrophil activity hypochlorous acid production by myeloperoxidase was determined by measuring accumulation of taurine chloramine [13]. Briefly, 10 μL of fresh heparinized blood samples or purified myeloperoxidase (10 nmol/L) was incubated with 10 nmol/L taurine in 10 nmol/L sodium phosphate buffer (pH 7.4) plus 140 mmol/L sodium chloride (PBS) at 21 °C for 5 min. The reaction was started by addition of 30 μmol/L hydrogen peroxide and stopped with 20 μg/ml catalase. The amount of taurine chloramine formed was assayed with 5-thio-2-nitrobenzoic acid. The activity of MPO was measured following the decrease of absorbance at 412 nm. One unit is the amount of MPO that can produce 1.0 nmole of taurine chloramine (hypochlorous acid) at pH 6.5 at 25 °C during 30 min in the presence of 100 mmol/L chloride and 100 mmol/L of hydrogen peroxide.

2.3. Bone analysis

2.3.1. Determination of ash content

Ash is the inorganic residue remaining after the water and organic matter have been removed by heating in the presence of oxidizing agents, which provides a measure of the total amount of minerals within a femur bone of five replicates. Femur bone samples of 1 g were used in the analysis of ash content. Bone samples were defatted by solvent extraction; this facilitates the release of the moisture and prevents spattering. Dry ashing procedures use a high temperature muffle furnace capable of maintaining temperatures of between 500 and 600 °C. Water and other volatile materials are vaporized and organic substances are burned in the presence of the oxygen in air to CO₂, H₂O and N₂. Most minerals are converted to oxides, sulfates, phosphates, chlorides or silicates. The ash content can be expressed on either a dry or a wet basis:

\[
\% \text{Ash (dry basis)} = \frac{M_{\text{ash}}}{M_{\text{dry}}} \times 100
\]

where \(M_{\text{ash}}\) refers to the mass of the ashed sample, and \(M_{\text{dry}}\) refers to the original masses of the dried and wet samples.

2.4. Determination of calcium and magnesium

Femur bones were separated critically dried at 100 °C for 24 h and ash was determined. A known weight of ash was dissolved in 1 ml concentrated nitric acid and completely digested in hot plate at 60 °C with regular added bi-distilled water and complete the volume to 5 ml in both control and experimental groups. Calcium and magnesium were measured by atomic absorption spectrophotometer [14].

2.5. X-ray analysis

Femur bones of both control and experimental groups were dried up to a critical point by a standard technique suitable for preparing samples for electron microscopy. Five bones of each group were chosen for the experiment. The dried samples were examined by a micro-CT desktop system, which based on the combination of X-ray projection microscopy with a tomographical reconstruction technique [15,16]. Accurate beam-hardening correction was implemented. Gray values in the virtual cross-sections were calibrated as calcium mineral density in bone. From these cross-sections, three-dimensional models were created. Minerals content were calculated directly from images and expressed as percentage per volume and per weight.

2.6. Immunohistochemistry

Samples of fumer were decalcified in 20% EDTA and embedded in paraffin wax as described previously [17]. Endogenous peroxidase activity was quenched by a H₂O₂/methanol wash, followed by hyalurondase treatment. Samples were blocked with goat serum in phosphate-buffered saline (PBS) for 1 h and incubated for 1 h with the primary antibody against phospho-FoxO1 (pSer256) (diluted 1:50). Afterwards, they were then incubated for 1 h with the relevant secondary antibody, biotinylated goat anti-rat IgG (F6258). Color was developed by incubation with DAB for 5 min; sides were then counterstained with H&E and mounted with Vecta-Mount.

2.7. Histological observations

Femur bones of both control and experimental groups were incised immediately at the end of treatment, fixed in 10% formal saline, followed by decalcification in 5% EDTA and dehydrated in ascending grades of ethyl alcohol, cleared in xylol and mounted in molten paraplast 58–62 °C. Five μm thick longitudinal histological sections were stained with hematoxylin and eisin and examined under bright field light microscope.

2.8. Duodenum alteration

2.8.1. Scanning electron microscope

About 1 mm slice of tissue from the middle portion of the duodenum, jejunum, ileum and cecum were fixed in 4% gluteraldehyde. Tissue samples for SEM were processed as described previously [18,19]. The lumen was cut open under the dissection microscope to select the right orientation before mounting on the stud. Specimens were dried in a critical point drying apparatus (BALTEC-SPD 030) using liquid carbon dioxide as the medium. Sputter-coated with gold (BALTEC-SCD 005 vacuum coater) at 100 ml. 7 mA for 3 min before being examined with a Jeol-SEM (JSM-6400, Japan) at 8 kV.
2.9. Western blot analysis

Twenty μg of duodenum tissue homogenate was added to lysis buffer containing 20 mM Tris–HC1 (pH 7.5), 1% Triton X-100, 137 mM sodium chloride, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 25 mM b-glycerophosphate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride and 1 g/ml leupeptin. Equal amounts of aliquots of tissue homogenate containing 60 μg of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein was then fractionated using a 10% separating gel. The fractionated proteins were then transferred electrophoretically to nitrocellulose paper, and the proteins were immunoblotted with specific antibody for phospho-PKB/AKT (pSer473) (diluted 1:1000) and incubated for 1 h and then at 4 °C overnight. After washing with Tris-buffered saline Tween-20, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody. The nitrocellulose papers were developed using 5-bromo-4-chloro-3-indolyl phosphate/4-nitroblue tetrazolium. Quantification of the bands was carried out using NIH Image J software. To ensure equal loading of the protein, β-actin was used as an endogenous control.

2.9.1. Data analysis

All data collected were subjected to analysis of variance among the groups using one way ANOVA. Significant differences between mean values were considered at p ≤ 0.05.

3. Results

3.1. Blood glucose alteration

Blood glucose level was recorded 178.00 ± 59.842 mg/dl, 573.26 ± 119.398 mg/dl and 225.61 ± 135.774 mg/dl in the control, diabetic and anti-TNF-α groups. Of note, the level of glyceremia was significantly higher by factors of 3.22 and 2.54 compared with control and anti-TNF-α (Fig. 1).

3.2. Femur bone alteration

To investigate the alteration in the femur, we have determined the most important minerals in bone based on biochemical reaction. Data show that diabetic rats exhibited a high decrease of calcium and ash content, meanwhile, magnesium content was markedly increased (Table 1). Accordingly, to verify our data, X-ray analysis on femur was carried out. Following X-ray analysis, the percentages of calcium and zinc content were markedly decreased, however, percentages of phosphorus and copper were increased (Table 2) in diabetic group. However, no significant difference was recorded when control and anti-TNF-α groups were compared, except for Zn, which significantly decreased in anti-TNF-α group. Histological analysis of bone of control group showed that the femoral epiphyseal region is formed of regularly arranged four zones: zone of resting cartilage composed of small scattered chondrocytes, zone of proliferation formed of regular arranged column of cartilage cells aligned near each other, zone of hypertrophied cartilage cells and zone of calcified cartilage degeneration and death of chondrocytes (Fig. 2a). In experimentally diabetic rat, the femoral head revealed a considerable thinning of femoral epiphyseal cartilage associated with marked degeneration of chondrocytes and loss of cartilage column. Fine collections of chondrocytes were sparsely distributed in the epiphyseal plate. Abnormal derangement of epiphyseal line was detected. The trabecular bone attained considerable thinning. A significant reduction in chondrocytes areas and numbers were noted in diabetes group compared with the rest groups. Since, chondrocytes areas were 15.80 ± 3.268 μm², 8.98 ± 1.427 μm² and 11.43 ± 2.582 μm² for control, diabetic and anti-TNF-α groups, respectively (Fig. 2b). The number of chondrocytes was highly significant decrease into 66.77 ± 7.679 μm² compared with control and treated group, which were recorded 91.454 ± 22.275 μm² and 82.68 ± 15.465 μm², respectively. FOXO1 expression was only detected in chondrocytes nucleus in form of brown stain, however, its expression was completely absent in chondrocytes nucleus in each of control and anti-TNF-α groups.

3.3. Duodenum alteration

In general, the duodenum is composed of four main distinctive regions: mucosa, submucosa, muscularis and adventitia. The mucosa is arranged into finger like projections called villi with intervening short glands called crypts. The peripheral surface of the villi is lined by a simple columnar epithelium. The goblet cells are regularly arranged in between the epithelial cells. The submucosa is enclosed by loose collagenous tissues and infiltrated by blood vessels. The muscularis is composed of dispersed smooth muscles. The adventitia represents the outermost cell layers and is formed of collagenous tissues outlined by serosa of simple squamous epithelium. Scanning electron micrographs of duodenum of rats in Fig. 3 showing that: in control group, normal distribution for mucus layer on the epithelium (A), well recognize finger-like shape (B), tall, spathulate villi with horizontally-arranged

![Fig. 1. Blood glucose level in blood plasma of rats.](image-url)

### Table 1

<table>
<thead>
<tr>
<th>Ash (g)</th>
<th>Mg (μmol/100 mg)</th>
<th>Ca (μmol/100 mg)</th>
<th>Subject</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2800 ± 0.005</td>
<td>0.2122 ± 0.006</td>
<td>1.73 ± 0.053</td>
<td>Control</td>
</tr>
<tr>
<td>0.3213 ± 0.006**</td>
<td>0.2656 ± 0.005**</td>
<td>1.416 ± 0.028*</td>
<td>Diabetic</td>
</tr>
<tr>
<td>0.253 ± 0.053a</td>
<td>0.1922 ± 0.07</td>
<td>1.61 ± 0.03</td>
<td>Anti-TNF-α</td>
</tr>
</tbody>
</table>

* p < 0.05.
** p < 0.01.
*** p < 0.001.

### Table 2

<table>
<thead>
<tr>
<th>Zn</th>
<th>Cu</th>
<th>Ca</th>
<th>P</th>
<th>Subject</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.9 ± 0.421</td>
<td>1.1 ± 0.385</td>
<td>78.8 ± 6.543</td>
<td>17.32 ± 0.2788</td>
<td>Control</td>
</tr>
<tr>
<td>2.6 ± 0.327</td>
<td>6.3 ± 1.262</td>
<td>12.9 ± 3.123</td>
<td>27.20 ± 0.4011</td>
<td>Diabetic</td>
</tr>
<tr>
<td>2.33 ± 0.801</td>
<td>2.19 ± 1.56</td>
<td>67.45 ± 7.46</td>
<td>22.32 ± 0.71</td>
<td>Anti-TNF-α</td>
</tr>
</tbody>
</table>

* p < 0.05.
** p < 0.01.
surface cleft (C), with well recognized goblet cells (D). The villi height reached to 220 μm with broad width of 110 μm in control group (C). In diabetic, degeneration of mucosal covering epithelium (A1) and jagged tips with protuberances were observed (B1). Dome-shaped cells and atrophied degenerative phases of their peripheral borders (C1) (an abrasion on the villus surface). Massive reduction of glandular opening of goblet cells (D1). The villi height reached to 195 μm with broad width of 85 μm manifesting more reduction comparing with control and anti-TNF-α treated groups (C1). In anti-TNF-α treated group, mucus probably coating the degenerated cells formed strands over the villi (Fig. 3A2). Villi still have broad finger-like shape (C1) with a slightly compressed laterally and recesses on the surface (B1). Goblet cells with their openings can be seen between the epithelial cells (D1). The villi height reached to 218 μm with broad width of 97 μm (Fig. 3C1). Histological analysis of duodenum (Fig. 4a) revealed dense leukocytes infiltration associated with villus core separation were detected, submucosal edema, reduction of crypts size sloughing of epithelial cells and massive reduction of goblet cells. Fainly alcin blue staining of goblet cells manifested reduction of their numbers within the villi. Since, the percentage ratio of goblet cells number (Fig. 4b) was significantly reduced in diabetic to 7.78 ± 2.336 when compared with control (11.91 ± 5.007) and anti-TNF-α (10.71 ± 3.016) groups (Fig. 5).

3.4. Cytokines profile

Data reflect a prominent decrease \((P < 0.001)\) in the level of IL-2 in diabetic group compared with control and anti-TNF-α groups. Since, the level of IL-2 was recorded 246.091 ± 17.249 Pg/gm, 295.770 ± 14.660 Pg/gm and 281.936 ± 15.577 Pg/gm in diabetic, control and anti-TNF-α groups, respectively. In contrast, the level of IL-6 was significantly \((P < 0.01)\) elevated in diabetic group (69.06 ± 3.27 Pg/gm) as compared with control (50.34 ± 4.82 Pg/gm) and the anti-TNF-α diabetic treated group (54.34 ± 4.82 Pg/gm). On the other hand, the level of TNF-α was prominently decline in the anti-TNF-α diabetic treated group (82.451 ± 11.88 Pg/gm) by factors 1.6~2 compared with control (131.618 ± 12.147 Pg/gm) and by factors ~2 compared with diabetic group (163.895 ± 14.135 Pg/gm). Their was a significant increase \((P < 0.01)\) in the level of TNF-α in diabetic group compared with control group.

3.5. Akt-1 phosphorylation

As a result of blocking of TNF-α, phosphorylation of AKT was increased by factors of 1.8 and 2.02 compared with control and diabetic groups, respectively (Fig. 6).

3.6. Mucosal immunoglobulin A

In diabetic group, the level of mucosal IgA was highly significantly decline \((p < 0.001)\) compared with control and anti-TNF-α groups. Since, data recorded 147.775 ± 2.466 μg/gm, 130.109 ± 1.533 μg/gm and 144.541 ± 2.792 for control, diabetic and anti-TNF-α groups, respectively. Their was also a significant difference \((p < 0.05)\) was noted when compared the anti-TNF-α group and control group (Fig. 7).

3.7. Caspase-3 activity

The activity of Caspase-3 was significantly increased in diabetic group (431.63 ± 27.13 U/mg) compared with control and the
Fig. 3. Scanning electron micrographs of duodenum of rats showing that: in control group, normal distribution for mucus layer on the epithelium (A), well recognize finger-like shape (B), tall, spatulate villi with horizontally-arranged surface cleft (C), with will recognized goblet cells (D). In diabetic, degeneration of mucosal covering epithelium (A1), jagged tips with protuberances were observed (B1). Dome-shaped cells and atrophied degenerative phases of their peripheral borders (C1) (an abrasion on the villus surface) and massive reduction of glandular opening of goblet cells (D1). In anti-TNF-α treated group, mucus probably coating the degenerated cells formed strands over the villi (A2). Villi still have broad finger-like shape (C1) with a slightly compressed laterally and recesses on the surface (B1). Goblet cells with their openings can be seen between the epithelial cells (D1).

Fig. 4. Alcian blue staining of duodenum of control (A), diabetic (B) and anti-TNF-α (C). Diabetic rats have a progressive decrease of the number of goblet cells (blue) along the small intestine. The number of goblet cells was counted in the duodenum (n = 5 rats).
anti-TNF-α groups, since, they were recorded 367.25 ± 23.57 U/mg, 385.25 ± 23.57 U/mg, respectively (Fig. 8).

3.8. Neutrophils activity

Myeloperoxidase (MPO) is the most abundant protein in neutrophils (also found in monocytes). Meloperoxidase can also use hydrogen peroxide resulting from the reduction of superoxide radicals to oxidize chloride to hypochlorous acid (HPO). It is important to clarify that the increased basal neutrophil MPO activity is not related to soluble plasma MPO due to intracellular MPO activity. In diabetic group, data show that the activity of myeloperoxidase was significantly increased by factors 1.43 and 2.25 compared with control and anti-TNF-α groups, respectively. Since, MPO was recorded 21.42 ± 3.68 U/ml, 48.22 ± 4.03 U/ml and 33.58 ± 3.42 U/ml for control, diabetic and anti-TNF-α groups, respectively (Fig. 9).

4. Discussion

IgA-producing plasma cells comprise ~20% of total plasma cells of peripheral lymphoid tissues, in human and mouse, whereas more than 80% of plasma cells produce IgA in mucosa-associated lymphoid tissues [20]. Secretory IgA is constitutively released into the lumen via the transport system of the intestinal epithelium [21]; it prevents invading pathogens from binding to mucosal epithelial cells and neutralizes their toxins [22,23]. In this study, a significant reduction in the mucosal IgA was recorded in diabetic group when compared whether with control or treated groups. Of note, IgA is the most abundant immunoglobulin in the body and has a central role in mucosal immunity [24,25]. That could explain the dramatic alteration and atrophied changes in the duodenum of diabetic rats. Since, in response to antigenic stimulation by the intestinal commensal bacteria, IgA release is induced from IgA-producing plasma cells [26]. As a result of anti-TNF-α injection, the level of basal mucosal immunoglobulin A was elevated by factor 1.09. This elevation in IgA may provide a stringent cellular protection for mucosal and epithelial cells from the destructive effect of different inflammatory and oxidized mediators. In this regard, a recent study by Hiroiuyki Tezuka et al. (2007) has been addressed one of the most biologically important and longstanding questions in immunology, which was why this ‘biased’ IgA synthesis takes place in the mucosa-associated lymphoid tissues (MALT), but not other lymphoid organs [20]. They showed that IgA class-switch recombination (CSR) is impaired in inducible-nitric-oxide (iNOS)-synthase-deficient mice [20]. Since, iNOS regulates the T-cell-dependent IgA CSR through expression of transforming growth factor-β receptor, and the T-cell-independent IgA CSR through production of a proliferation-inducing ligand and a B-cell-activating factor of the tumor necrosis factor (TNF) family.

On the other hand, it has been established that IL-2 plays a pivotal role in programming T cells for activation-induced cell death. IL-2 played an additional role in immune regulation, exogenous IL-2 distraction could reverse the excessive death of isolated CD25+ T cells and restored their proliferative activity [27]. Surprisingly, delivery of IL-2 a few days after birth resulted in low efficiency of disease improvement [28], therefore, IL-2 had to be provided at very early stages of CD25+ T dysfunction. In this study, the level of IL-2 was significantly decreased in diabetic group compared with other groups. As a result of targeting TNF-α their still was a significant decrease in IL-2 levels compared with control group, however, this decrease confer a minor effect compared with diabetic group.

Since, oxidative stress has been widely emerged for the pathogenesis of several diseases; we here in this study tried to investigate the activity of the major promoter for the reactive species production in order to understand consequence of events. Data show that the activity of myeloperoxidase (MPO) in whole blood was significantly higher in diabetic group compared with control and treated groups. That could put a finger on the crucial role of hyper neutrophils activity in diabetic complications. It is important to clarify that the increased basal neutrophil MPO activity is not related to soluble plasma MPO due to intracellular MPO activity [29]. In this context, it is very important to mention a fact that in order to eliminate functionless cells, IL-6 is the central inducer for neutrophil activation under stress conditions.

**Fig. 5.** Biochemical changes of IL-2, IL-6 and TNF-α expressed as Pg/gm.

**Fig. 6.** Total proteins were extracted and subjected to Western blotting using antibody directed against Akt-1 protein kinase. Protein levels of phosphorylated Akt-1 (p-AKT-1), or as a loading control beta actin.

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Therefore, the proinflammatory IL-6 could predict more precisely the severity and duration of inflammation, particularly in its acute phase than TNF-α [30]. As a result of the increased levels of reactive oxygen species in diabetic group, a prominent expression for the transcription factor FOXO1 has noted in the nucleus of chondrocytes, since, oxidative stress is the main mediator for FOXO1 upregulation. In general, FOXO1 is activated in order to eliminate oxidative stress hazard effects [31,32]. Furthermore, during periods of oxidative stress, FoxO transcription factors can lead to apoptosis [33]. Thus, the elevation of FOXO1 expression could explain the augmented levels of caspase 3 in diabetic group. In this concern, the increased levels of TNF-α may also be responsible for the increased cleaved caspase 3 levels in the diabetic group. Of note, there was a prominent reduction in chondrocytes areas and numbers along with prominent apoptotic changes in diabetic group, thus, TNF-α up-regulation could involve in such events. Data show that TNF-α up-regulation enhances susceptibility of minerals resorption in cartilage and bone. Additionally, upregulation of FOXO1 by TNF-α has been involved in pro-apoptotic alteration. In this regard, it has been proposed that conditions with prolonged or high levels of FOXO1 activation may be deleterious by inducing apoptosis [34,35].

On the other hand, it has been concluded that Akt mediates the physiological responses of insulin on glucose uptake [36] and glycogen synthesis [37]. In this study protein kinase B (Akt) phosphorylation was significantly decreased in diabetic group compared with control and anti-TNF-α treated groups. In the contrary, as a result of down-regulation of TNF-α by the specific antibody phosphorylation of Akt-1 was induced in treated group, thus in turn, a significant decrease in the level of the executor caspase-3 was noted. Since, Akt typically inhibits the cleavage of caspase 3, and thus, it could prevent the incidence of apoptosis [38]. That could emerge as a reason for apoptotic events in diabetic group. Additionally, it is very important to state that there is a reverse relation between the activation of Akt-1 and the presence or absence of FoxO1 in the nucleus [39]. On phosphorylation by Akt-1, Foxo1 is transported out of the nucleus due to phosphorylation. Therefore, FOXO1 expression has noted in chondrocytes nucleiuses of the diabetic rats, due to TNF-α upregulation and reactive oxygen species produced by neutrophils.

In conclusion, data of this study confirmed that the elevated levels of TNF-α upregulate FOXO1 expression in chondrocytes cells, which in turn, accelerate cartilage resorption, decrease cartilage areas and numbers. Therefore, over expression of TNF-α could involve in activation of transcription factor FOXO1 along with oxidative stress induced by neutrophils, which activated by the proinflammatory cytokine 6 (IL-6). Impairment in interleukin 2 (IL-2) and mucosal immunoglobulin A secretions in diabetic patients may be the main factors for the digestive tract atrophy and origins for inflammatory bowel disease.

Conflict of interest statement

None.

Acknowledgments

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