Immunohistochemical expression of visfatin in the gingival tissue of type II diabetes patients

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Abstract
Visfatin found to be associated with different systemic inflammatory diseases like diabetes and diseases of oral cavity like periodontal diseases so that the aim of this study to evaluate the expression of visfatin in the gingival tissue of control and uncontrolled type 2 diabetic patients. 28 type 2 diabetic patients divided into two groups, the first group consist of 18 control diabetic patients under hypoglycemic drug with chronic periodontitis, the second group which also consist of 18 patients but an uncontrolled diabetic (without hypoglycemic drug) and with chronic periodontitis and the third group 10 person with healthy periodontium. Their age was between (30-45) years and all patients in both groups were male and non-smokers. The periodontal condition was estimated by recording the plaque index, bleeding on probing, probing pocket depth and clinical attachment level. Gingival pocket epithelium samples were excised crown lengthening procedure and periodontal flap surgery in the diabetic group and healthy groups and then processed for hematoxylin and eosin staining then immunohistochemical staining for visfatin marker expression. Statistical analysis was done by using SPSS software and paired t-test, Mann-Whitney test. Statistically significant was considered at a p-value less than 0.05. The mean value of all recorded periodontal parameters was highest among uncontrolled diabetic group compared to control with statistically significant difference existed between both groups (p=0.000) for plaque index, bleeding on probing, probing pocket depth and clinical attachment level. In regard to the immunohistochemistry staining, the Mann-Whitney test was used and the results revealed significant differences between the diabetic groups (p= 0.000). In conclusion, the study revealed the poor condition of the oral cavity regarding the periodontal condition of patients with type 2 diabetes, so the cooperation between diabetic centers and dentists needs to be increased. The immunohistochemical stain for visfatin was found more intense in the gingival tissue of the uncontrolled diabetic group compared to the control diabetic one. So that visfatin may have a link to the etiology and pathology of type 2 diabetes and chronic periodontitis.

Key words: Type II diabetes, visfatin, immunohistochemistry

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Introduction
Diabetes Mellitus (DM) can be included as a clinical and genetic, heterogeneous disease that affects the carbohydrates, proteins and lipids metabolism resulting in hyperglycemia (1). Diabetes is present differently, but type two diabetes mellitus (T2DM) is predominant. 90% of cases are type 2. It is thought that about 382 million people around the world are affected. By the year 2035, this quantity expected to increase to 592 million. Furthermore, the frequency proportions of T2DM are considered to be more in males (8.4%) than females (7.1%). The cause of this great ratio is considered a serious situation for status by elevating the morbidity and mortality of the people affected in both western and developing countries (2).

The hyperglycemic situation if uncontrolled may result in activation of several pathways, such as advanced glycationendproducts (AGEs), polyol pathways, increase range of protein kinase C (PKC) action and hexosamine biosynthesis pathways and this will aggravate the situation. The dangerous problems are demonstrated by retina, neuro, nephron, and angiopathologies also included atherosclerosis and periodontitis. T2DM enhances the inflammatory responses of the periodontal tissue toward pathogens, causing the risk susceptibility of periodontal tissue disease two to five times when compared to the non-diabetic persons (3).

Nicotinamide phosphoribosyltransferase (NAMPT) also known as visfatin, is a pre-B-cell colony-enhancing factor. The production of visfatin is mainly by macrophages and adipocytes (4). Furthermore, visfatin can be detected in cells such as monocytes from the peripheral blood dendritic cells, and lymphocytes. It has the ability to release multi mediators of inflammation such as interleukin-6 (IL-6), IL-1β, and tumor necrosis factor-alpha (5-6).

Visfatin has been detected in metabolic syndromes, inflammatory diseases, diabetes, atherosclerosis, and also in cancers (7-8). Several meta-analyses showed the association of periodontitis with different diseases such as type 2 diabetes and cardiovascular diseases (9-10). Recent research has reported visfatin levels to rises in saliva, serum, gingival crevicular fluid (GCF), and periodontal ligament in persons with periodontitis and this can lead to the role of visfatin in the pathogenesis of those diseases. Therefore, it could be possible that visfatin is the pathomechanistic link between periodontitis with some systemic diseases (11-12).

Materials and methods
Sample
The study group consisted of (28 males, non-smokers) with a confirmed diagnosis of type 2 diabetes. The study group further subdivided into 2 groups according to their control of the glucose level into 14 Patients under hypoglycemic agents (control diabetic) and 14 patients without medication (uncontrolled diabetic), patients under other medication and/or with any other systemic disease were excluded from this study. Their age was ranged between (30-45 years).

Periodontal clinical examination
Periodontal clinical parameters were carried out for all permanent teeth and four surfaces of each tooth were scored by using mouth mirror, diagnostic probe, and periodontal probes. The following indices were recorded: A) Plaque index (13). B) Bleeding on Probing. C) Probing Pocket Depth. D) Loss of Attachment

Immunohistochemistry
Patients Selection
Gingival tissue samples from 28 of human patients were used for this study. All patients included in this study had presented for routine oral surgery or periodontal surgery and the tissue samples collected were gingival tissue normally excised and typically discarded as part of the standard of care in this type of treatment. Controls diabetic and uncontrolled diabetic patients were confirmed using a written health questionnaire and an oral interview. Individuals meeting the inclusion criteria were asked to participate in this study and given the informed consent form to sign. All of the test subjects had Type 2 diabetes mellitus.

Tissue Samples
Samples from periodontal tissue were gotten throughout crown lengthening and periodontal flap surgery with an estimated 1.5–3 mm in thickness and 1-2 mm in height in diabetic group patients. After giving local anesthesia (2% lidocaine with 1:100,000 epinephrine), pocket epithelial samples from places with a probing pocket depth of 5 mm or more, clinical loss of attachment at least 3 mm, and the existence of bleeding on probing were removed by secuals and internal bevel incisions using blade no. 15, 12.

Immunohistochemical steps
Immunohistochemical staining in the standard envisions, slices were mounted on poly-L-lysine-coated slides. After deparaffinization and rehydration, the sections were incubated in 0.01M citrate buffer in a microwave oven for antigen retrieval. Washing the slides in phosphate-buffered saline (PBS) and incubated in 0.5% H2O2 in methanol to block endogenous peroxidase activity. Primary - Rabbit Anti-Visfatin antibody (ab58640) for 1 h at room temperature was applied. After washing in PBS for 5 min, the pieces were incubated with a secondary antibody to improve the sensitivity of the procedure; Goat Anti-Rabbit IgG H&L (HRP) (ab205718) for a half-hour at room temperature. After washing with PBS, the immunoreactivity was visualized by diaminobenzidine hydrochloride as the chromogen for five minutes. Slices were lastly counterstained with hematoxylin, cleared, and mounted with PV mount. Negative controls consisted of PBS instead of primary antibody and samples of positive controls were human kidney tissue. Then, the tissue sections were scanned under the light microscope in 10 arbitrarily selected high power (x400) fields by a pathologist who had no prior knowledge of the patient’s clinical status. Immunohistochemical staining for visfatin was scored based on the percentage and the intensity of positively cytoplasmic stained cells. The five score categories for positive staining percentage were as follows: 0, no positive cells; 1, 25% or fewer positive cells; 2, 26%
to 50% positive cells; 3, 51% to 75% positive cells; and 4, 76% or more positive cells. Scoring of the intensity of staining was as follows: 0, no intensity; 1, weak intensity; 2, moderate intensity; and 3, strong intensity. Visfatin expression was determined by adding the positive staining percentage score and intensity score” (14). Table (1) shows the distribution of data according to grades.

Results
A significant difference statistically for the periodontal parameters (PII, BOP, and PPD, CAL) between the control diabetic group and the uncontrolled diabetic group, t-test was used and the result appears in the table (2). Figure (1) shows the staining with Hematoxylin and Eosin in the gingival tissue of healthy tissue, control diabetic gingival tissue and uncontrolled diabetic gingival tissue while Figure (2) shows visfatin immunohistochemical staining in the gingival tissue of healthy tissue, control diabetic gingival tissue and uncontrolled diabetic gingival tissue. Table (3) shows the statistical analysis for non-parametric sample, Mann-Whitney Test was used and the results shown a significant differences for visfatin expression in the gingival tissue between control diabetic and uncontrolled diabetic samples with p=0.00.

Table (1): Distribution of data according to grades.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Control diabetic</th>
<th>Uncontrolled diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade I</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Grade II</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Grade III</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Total number of sample</td>
<td>14</td>
<td>14</td>
</tr>
</tbody>
</table>

Table (2): t–test of periodontal parameters among control group and uncontrolled diabetic group.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control Diabetic Group</th>
<th>Uncontrolled Diabetic Group</th>
<th>t–value</th>
<th>p–value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>+ SD</td>
<td>Mean</td>
<td>+ SD</td>
<td></td>
</tr>
<tr>
<td>PII</td>
<td>1.0620</td>
<td>1.9545</td>
<td>-6.943</td>
<td>0.000*</td>
</tr>
<tr>
<td>BOP</td>
<td>0.3660</td>
<td>0.8125</td>
<td>-7.431</td>
<td>0.000*</td>
</tr>
<tr>
<td>PPD(mm)</td>
<td>3.7900</td>
<td>5.0800</td>
<td>-4.888</td>
<td>0.000*</td>
</tr>
<tr>
<td>CAL(mm)</td>
<td>1.860</td>
<td>3.355</td>
<td>-7.351</td>
<td>0.000*</td>
</tr>
</tbody>
</table>

Figure (1): Staining with Hematoxylin and Eosin in the gingival tissue of (a) healthy tissue, (b) control diabetic gingival tissue, (c) uncontrolled diabetic gingival tissue (x10).
**Figure (2):** visfatin immunohistochemical staining in the gingival tissue of (a) healthy tissue, (b) control diabetic gingival tissue, (c) uncontrolled diabetic gingival tissue (x10).

**Discussion**

In the present study, the observation of the result in regard to periodontal parameters show that poor oral hygiene and level of plaque index, bleeding on probing, probing pocket depth and clinical attachment loss were significantly higher among the uncontrolled diabetic group in comparison to control diabetic patients. A relationship of visfatin with a variety of diseases as (diabetes atherosclerosis, metabolic syndromes, obesity, and cancers has been reported. Several meta-analysis studies showed that periodontitis is associated with other diseases as (obesity, type II diabetes, metabolic syndrome, cardiovascular diseases, bowel inflammatory disease, and rheumatoid arthritis) \(^{15}\). Recently, visfatin levels increase in (serum, gingival crevicular fluid (GCF), saliva, and periodontal tissues) in chronic periodontitis patients and play a role in the pathogenesis of the disease. Visfatin could be the probable pathomechanistic connection between periodontitis and certain systemic diseases. Synthesis of visfatin is stimulated in the periodontium by pathogens responsible for periodontal pathology as (*Porphyromonas gingivalis* and *Fusobacterium nucleatum*) and some cytokines as IL-1\(^\beta\)\(^{16,17}\).

Our study estimates the visfatin existence and spread in the gingival tissues by using the immunohistochemistry staining technique of two groups, the first group controlled type 2 diabetic with chronic periodontitis( under hypoglycemic drug), and the second group was uncontrolled diabetic with chronic periodontitis( without medication). The results by using the Mann-Whitney test revealed a high expression of visfatin in the uncontrolled diabetic patients with a significant difference between the two groups. The explanation of these results is that visfatin can mimic insulin functions and has pro-inflammatory effects and dysregulation of the action of visfatin subscribe to diabetes and metabolic syndrome, also the existence of periodontal diseases (chronic periodontitis) increase the pro-inflammatory cytokines and one of this visfatin which impact insulin resistance and modify inflammation and immune responses. No previous study was done to evaluate the expression of visfatin in the gingival tissue by using immunohistochemistry for such groups.

The previous study was done which evaluate the expression of visfatin in the gingival tissue between chronic and aggressive periodontitis and the result reveal a statistically significant and strong association between these groups and more visfatin expression was found in aggressive periodontitis group in comparison to chronic periodontitis group. In conclusion, Poor oral hygiene and severe periodontitis are more commonly found in uncontrolled diabetic patients with high HbA1c levels. A significant difference in the expression of visfatin marker in the gingival tissue between control and uncontrolled diabetic groups, so that co-operation between a specialist in diabetic disease and dentists needs to be intensified.

**References**
