RELATIONSHIP BETWEEN SERUM PROTEIN CARBONYL CONTENT, TOTAL THIOL AND GLYCATED HEMOGLOBIN IN EGYPTIAN TYPE-2 DIABETIC PATIENTS

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ABSTRACT

Hyperglycemia is one of the most important factors that are responsible for oxidative stress and production of reactive oxygen species (ROS) in diabetes. Proteins are likely to be major targets of ROS, as a result of their abundance in cells, plasma, and most tissues, and their rapid rates of reaction both with many radicals and with other oxidants. Proteins that are damaged by oxidative stress have decreased biological activity leading to loss of energy metabolism, cell signaling, transport, and, ultimately, to cell death. Protein carbonyl content is the most general and well-used biomarker of severe oxidative protein damage.

Aims:
To investigate the relationship between serum protein carbonyl content, total thiol and glycated hemoglobin (HbA₁c) in Egyptian patients with type 2 diabetes mellitus under poor glycemic control.

Methods:
Fifty patients with type 2 diabetes mellitus were recruited from the department of Internal medicine, Sohag University Hospital, Egypt and 20 age- and sex-matched healthy controls. Serum protein carbonyl content, total thiol and HbA₁c were measured.

Results:
The study revealed that serum protein carbonyl content in diabetic patients was significantly higher than the controls (19.9 ± 5.3 vs. 2.43 ± 1.5 nmol/L, \(P < 0.001\)), while total thiol was significantly lower in the patients than the controls (193 ± 50.6 vs. 298 ± 78.5 μmol/ml, \(P < 0.001\)). Protein carbonyl content was negatively correlated to total thiol, positively correlated to HbA₁c and total thiol was negatively correlated with HbA₁c.

Conclusions:
Hyperglycemia is associated with an increase in the protein oxidation in terms of an increase in protein carbonyl content and a decrease in total thiol group. Therefore, poorly controlled diabetic patients may be subjected to numerous pathological conditions related to the increased protein oxidation.
INTRODUCTION

Diabetes Mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. (American Diabetes Association, 2010). In 2011 there were 366 million people with diabetes, and this is expected to rise to 552 million by 2030. Most people with diabetes live in low- and middle-income countries, and these countries will also see the greatest increase over the next 19 years (Whiting et al., 2011).

Under normal physiological conditions, there is a balance in the generation of oxygen-free radicals and the antioxidant defense mechanisms used to deactivate free radical toxicity. Impairment in the oxidant/antioxidant equilibrium results in oxidative stress in numerous pathological conditions including diabetes leading to cellular damage (Zheng and Kern, 2009). Increasing evidence in both experimental and clinical studies suggests that there is a close link between hyperglycemia, oxidative stress, and diabetic complications (Wu et al., 2009). Oxidative stress due to hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart and blood vessels. Oxidative stress induced by hyperglycemia is involved in both the development and progression of the disease and can lead to serious complications such as blindness, kidney damage, lower limb amputations, and cardiovascular diseases. Oxidative stress in DM causes also several adverse effects on the cellular physiology where it is particularly relevant and critical for those tissues that have lower levels of intrinsic antioxidant defenses (Evans et al., 2003).

Proteins are likely to be major targets of ROS, as a result of their abundance in cells, plasma, and most tissues, and their rapid rates of reaction both with many radicals and with other oxidants. Attack of ROS modifies amino acid; lysine, arginine, proline, and histidine residues generating carbonyl moieties, chloraminated oxidants, mainly hypochlorous acid and chloramines, produced by myeloperoxidase in activated neutrophils, form dityrosine containing cross-linked protein products known as advanced oxidation protein products (AOPPs), both of which have been identified as an early marker for oxidative stress and are used as a measure of protein damage (Witko-Sarsat et al., 1996).

Studies have showed proteins that are damaged by oxidative stress have decreased biological activity leading to loss of energy metabolism, cell signaling, transport, and, ultimately, to cell death (Vincent et al., 2004). Those oxidative stress induced damages have been demonstrated on cell based (Askwith et al., 2009), in vivo animals (Schmeichel et al., 2003), and human clinical studies (Ziegler et al., 2004). Under clinical observations, the impaired glucose tolerance and advanced glycation end products are positively associated with the development and progress of the oxidative stress. As a result, new therapies are aimed at the underlying pathogenesis as well as the symptom complex (Goh and Cooper, 2008).

The glycated hemoglobin ($HbA_{1c}$) concentration is a measure of the 2–3 months average endogenous exposure to glucose. It has low intra-individual variability and can be determined in the non-fasting state (Selvin et al., 2007). Therefore, $HbA_{1c}$ is used for the estimation of glucose control in subjects with known diabetes (American Diabetes Association, 2009). Recently, $HbA_{1c}$ has also been included in the diagnosis algorithm of diabetes because it indicates the risk of microvascular disease (Silbernagel et al., 2011).
Carbonyl (CO), aldehyde or ketone groups, are produced on protein side chains when they are oxidized. Protein carbonyl content is the most general and well-used biomarker of severe oxidative protein damage. Human diseases associated with protein carbonylation include Alzheimer's disease, chronic lung disease, chronic renal failure, diabetes and sepsis. Furthermore, CO groups may be introduced into proteins by a secondary reaction of the protein side chains with reactive carbonyl derivatives generated as a consequence of the reaction of reducing sugar with lysine residues of proteins with the eventual formation of advanced glycation end products such as pentosidine or carboxymethyllysine (Dall-Donne et al., 2003).

Thiol compounds contain a sulfhydryl group (-SH) attached to the carbon atom. Human plasma contains homocysteine (HcySH), cysteinylglycine (CysGlySH), cysteine (CysSH) and glutathione (GSH) as reduced thiols (Rossi et al., 2009). Thiol groups are also present on plasma proteins especially albumin (Prakash et al., 2004). Cys-34 of albumin accounts for the bulk of free thiol in plasma. Thiols assist aerobic cells to maintain a reducing state, despite an oxidizing environment (Chung et al., 2005). Protein sulfydryl group is a good reflection of excess free radical generation, since the conformation of albumin is altered, allowing –SH groups to be oxidized (Cakatay and Kayali, 2005).

The aim of this study is to investigate the relationship between serum protein carbonyl content, total thiol and HbA1c in Egyptian patients with type 2 DM under poor glycemic control.

PATIENTS AND METHODS:

**Patients:** Fifty patients (22 males and 28 females) with type 2 DM according to the criteria of the Expert Committee (Expert Committee, 1997) were recruited from the department of Internal medicine, Sohag University Hospital. All patients were on oral hypoglycemic drug therapy and their HbA1c more than 7%, those taking insulin therapy or those with HbA1c less than 7%, were excluded from the study. The clinical characteristics of the patients were indicated in Table 1.

**Controls:** Twenty healthy control subjects (9 males and 11 females), their age and sex distribution were similar to those of the patients (Table 1). They were recruited from the hospital staff. All volunteers (diabetic patients and normal subjects) were informed about the nature of the study; the subjects gave written consent for the use of their blood samples for scientific study. The protocol of study was in conformity with the guidelines of the Sohag University Ethical Committee.

**Collection of blood:** three ml of venous blood were obtained by venipuncture from the patients and controls under fasting conditions and centrifuged immediately. Sera were frozen in aliquots and stored at -20 °C till time of assay.

**Chemicals:** Guanidine HCl (product #: SRE0066), 5,5′-dithiobis-2-nitrobenzoic acid (product #: D8130) and 2,4-dinitrophenylhydrazine (product #: D199303) were purchased from Sigma-Aldrich (St. Louis, MO. USA). Fasting blood glucose and HbA1c were performed for all patients and controls using Cobas C 311 analyzer, Roche diagnostics, Germany.

**Determination of protein carbonyl content:** Protein carbonyls content was measured according to procedure of Levine et al. (1990). Protein carbonyl reacts with 2,4-dinitrophenylhydrazine (DNPH) to generate chromophoric dinitrophenylhydrazones. Proteins were
then precipitated by trichloroacetic acid (TCA) and the free DNPH is removed by washing the protein pellet. The protein pellet was dissolved in 6 M guanidine HCl and the absorbance of protein-hydrozone was measured spectrophotometrically at 370 nm.

Two tubes of 0.5 ml serum were used; one was marked as "test" and the other as "control". 2.0 ml of 10 mM DNPH prepared in 2.5 M HCl was added to the test and 2.0 ml of 2.5 M HCl alone was added to the control. The contents were mixed thoroughly and incubated in the dark at room temperature for 1 hour. The tubes were shaken intermittently every 15 min. Next, 2.5 ml of 20% TCA (w/v) were added to both tubes and the mixture was left on ice for 10 min. The tubes were centrifuged at 3,500 rpm for 20 min to harvest the protein pellet. Supernatant was carefully aspirated and discarded. This was followed by a second wash with 10% TCA as described above. Finally, the precipitates were washed three times with 2 ml ethanol:ethyl acetate (1:1 v/v) to remove unreacted DNPH and lipid remnants. The final protein pellet was dissolved in 1 ml of 6 M Guanidine HCl and incubated at 37°C for 10 min. The insoluble materials were removed by centrifugation.

Carbonyl concentrations were determined from the difference in absorbance at 370 nm between the test and the control using molar extinction coefficient of 22000 M⁻¹cm⁻¹. Carbonyl levels were expressed as nmol/L of serum.

**Determination of thiol group:** The serum –SH group was estimated according to (Hu, 1994). It is based on the ability of the –SH group to reduce Ellman' reagent (5,5′-dithiobis-2-nitrobenzoic acid or DTNB) and form a yellow colored anionic product whose OD is measured spectrophotometrically at 412 nm. 50 μl of the sample mixed with 1 ml of (0.1 M Tris, 10mM EDTA PH 8.2), constituting the blank reaction and assessed at 412 nm. Afterwards, 40 μl of 10 mM DTNB were added and the absorption read at 412 nm after stable color formation (1-3 minutes). The concentration of thiol groups were calculated using a molar extinction coefficient of 13.600 M⁻¹cm⁻¹. Thiol concentrations were expressed as μmol/ml.

**Statistical analysis:** Data were expressed as mean ± standard deviation. All analyses, descriptive statistics and graphics were performed using GraphPad Prism 2007 (GraphPad Software, San Diego, CA, USA). Differences between variables were assessed by Mann–Whitney test and were considered statistically significant at \( P < 0.05 \). Correlations among variables were tested using spearman correlation test.

**RESULTS:**

The clinical and laboratory characters of the patients are represented in Table 1. Serum protein carbonyl contents were significantly higher in diabetic patients than the controls (19.9 ± 5.3 vs. 2.43 ± 1.5 nmol/L, \( P < 0.001 \)), **Fig. 1.** Total thiol group decreased significantly in diabetic patients than in the controls (193 ± 50.6 vs. 298 ±78.5 μmol/ml, \( P < 0.001 \)), **(Fig. 2).**

Protein carbonyl contents were negatively correlated to total thiol (\( r = -0.796, \ P < 0.0001 \)), positively correlated to HbA₁c (\( r = 0.839, \ P < 0.0001 \)) and total thiol was negatively correlated with HbA₁c (\( r = -0.805, \ P < 0.0001 \)). **(Table 2).**
Table 1: Clinical and laboratory characters of controls and patients

<table>
<thead>
<tr>
<th>Character</th>
<th>Control (n = 20)</th>
<th>Patients (n = 50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>54.0 ± 8.60</td>
<td>53.4 ± 9.4</td>
</tr>
<tr>
<td>Duration of disease (years)</td>
<td></td>
<td>7.7 ± 2.6</td>
</tr>
<tr>
<td>Blood glucose (mg/dl)</td>
<td>93.7 ± 10.3</td>
<td>183 ± 10.7</td>
</tr>
<tr>
<td>HbA1C (%)</td>
<td>5.30 ± 1.33</td>
<td>10.9 ± 1.42</td>
</tr>
<tr>
<td>Protein carbonyl (nmol/L)</td>
<td>2.43 ± 1.5</td>
<td>19.9 ± 5.30</td>
</tr>
<tr>
<td>Total thiol (µmol/ml)</td>
<td>298 ± 78.5</td>
<td>193 ± 50.6</td>
</tr>
</tbody>
</table>

Table 2: Spearman correlation analysis in type 2 diabetic patients

<table>
<thead>
<tr>
<th>Parameters</th>
<th>r values</th>
<th>p values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein carbonyl content and total thiol</td>
<td>r = - 0.796</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Protein carbonyl content and HbA1c</td>
<td>r = 0.839</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Total thiol and HbA1c</td>
<td>r = - 0.805</td>
<td>p &lt; 0.0001</td>
</tr>
</tbody>
</table>

Figure 1: Mean ± SD levels of protein carbonyl in type 2 DM and controls (nmol/L)

Figure 2: Mean ± SD levels of total thiol in type 2 DM patients and controls (µmol/ml)
DISCUSSION:

In the present study, the relationship between serum protein carbonyl content, total thiol and HbA1c in Egyptian patients with type 2 DM under poor glycemic control was evaluated. The investigation showed that protein carbonyl content was significantly higher in diabetic patients than controls ($P < 0.001$), while total thiol content in diabetic patients was significantly lower than controls ($P < 0.001$). This study also showed a positive correlation between protein carbonyl content and HbA1c, while total thiol groups concentration was negatively correlated to both HbA1c and protein carbonyl content.

It was found that hyperglycemia induce mitochondrial superoxide production which, in turn activates four damaging pathways in cells through inhibiting the activity of the key glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase. Increased levels of glyceraldehyde-3-phosphate activate advanced glycation end product pathway, protein kinase C pathway, hexosamine pathway and polyol pathway (Brownlee, 2001). Moreover, the hyperglycemia induces effects within the cell nucleus through ROS. Hyperglycemia initiates a cascade of transcription events, ultimately leading to changes in the levels of NO, cytokines, acute-phase reactants and cellular adhesion molecules. Generation of ROS can be reduced by avoiding hyperglycaemia and by minimizing fluctuations in blood glucose levels (Wright et al., 2006).

Protein carbonyl content provides a global index of protein oxidation involving the side chains of several amino acid residues. They represent the stable end product generated upon formation of transient radical species, such as chloramines and nitrogen/carbon radicals, which are induced by oxidant stimuli. However, direct oxidation of amino acid side chain is not the only way through which carbonyl groups can be formed in proteins. In fact, glycation may induce formation of protein carbonyls, such as ketoamine derivatives, thus generating reactive radicals and perpetuating a vicious cycle (Levine et al., 1990).

Previous studies found significant increase in protein carbonyl in diabetic patients (Telci et al., 2000, Stadtman and Levine, 2003, Çakatay, 2005, and Arif et al., 2010). While Odetti et al. could not find any significant change in plasma protein, carbonyl levels between type 2 diabetic patients and controls (Odetti et al. 1999).

The maintenance of protein redox status is of fundamental importance for cell function, therefore structural changes in proteins are considered to be among the molecular mechanisms leading to diabetic complications. Alterations in protein conformations can lead to increased aggregation, fragmentation, distortion of secondary and tertiary structure, susceptibility to proteolysis, and decrease of normal function (Altomare et al., 1997 and Butterfield et al., 1998).

Thiol groups that exist both intracellularly and extracellularly either in free form or bound to proteins play a major role in maintaining the antioxidant status of the body (Rossi et al., 2009). Thiol status has been determined in both type 1 and type 2 DM and the levels were found to be decreased (Çakatay, 2005, and Arif et al., 2010). These decreases were partially explained by metabolic-, inflammatory- and iron alterations (Van Campenhout et al., 2006). A significant increase in free iron in the ferric state was found in diabetic patients under poor glycemic control which may explain the decrease in protein thiols (Shetty et al., 2008). The fact that thiols are facile
targets of glycation, and low molecular mass thiols are potent glycation inhibitors, aids the design of therapeutic agents for the treatment of the complications of diabetes (Zeng and Davies, 2006).

The inverse relation of plasma thiol group with protein carbonyl concentration in diabetic patients is a direct evidence of increased protein oxidation under conditions of elevated oxidative stress during diabetes; this may be correlated with altered glucose homeostasis and other late vascular complications in diabetes. Our results suggest that protein oxidation in type 2 diabetic patients might be promoted by an insufficient counter-regulation of the antioxidant system. The antioxidant system may therefore play a role in protecting against glycosylated damage by glycaemia induced oxidative stress.

CONCLUSION:

Hyperglycemia is associated with an increase in protein oxidation in terms of an increase in protein carbonyl content and a decrease in total thiol group. Therefore, poorly controlled diabetic patients may be subjected to numerous pathological conditions related to the increased protein oxidation.

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الملخص العربي

العلاقة بين محتوى مصل الدم من الكاربوين البروتيني، إجمالي الثيول والهيموجلوبين السكري في المرضى المصريين المصابين بالنوع الثاني من داء السكري

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قسم البيولوجيا - كلية الطب - جامعة سوهاج - مصر

فرت السكر في الدم هو أحد العوامل المهمة المسؤولة عن الأكسدة وزيادة الشوارد الحرة في مرضى السكري. وتشير البروتينات أكثر المركبات عرضة تأثير هذه الشوارد الحرة نظراً لوفرتها في الخلايا البيضاء، ومعظم الأنسجة وتفاعلها السريع مع عوامل الأكسدة، والبروتينات التي تتعرض للأكسدة فقد نشاطها البيولوجي والوظيفي مما يؤدي في النهاية إلى موت الخلايا.

الكاربوين البروتيني في الدم هو أهم علامات تأكسد البروتين. لذا أجري هذا البحث لدراسة العلاقة بين الكاربوين البروتيني، إجمالي الثيول والهيموجلوبين السكري في المرضى المصريين المصابين بالنوع الثاني من داء السكري.

اجريت هذه الدراسة على 50 مريض بداء السكري من النوع الثاني (22 ذكر و 28 إناث) و 10 شخص من الأصحاء (9 ذكر و 1 امرأة) في مستشفى كلية الطب جامعة سوهاج. تم سحب عينات الدم واستخلاص المصل منها لقياس الكاربوين البروتيني، إجمالي الثيول والهيموجلوبين السكري.

أظهرت النتائج زيادة ذو دلالة إحصائية في محتوى مصل الدم من الكاربوين البروتيني في مرضى السكري عن الأشخاص الأصحاء، وانخفاض ذو دلالة إحصائية في إجمالي الثيول في مرضى السكري عن الأشخاص الأصحاء، كذلك وجدت علاقة طردية بين الكاربوين البروتيني والهيموجلوبين السكري وعلاقة عكسية بين إجمالي الثيول والهيموجلوبين السكري.

من هذه الدراسة نستنتج أن ارتفاع السكر في الدم يؤدي إلى زيادة في كمية الكاربوين البروتيني وانخفاض في إجمالي الثيول، بينما قد يتعرض مرضى السكري إلى العديد من الحالات المرتبطة بزيادة أكسدة البروتينات.