Level of alpha dicarbonyl compound (methylglyoxal, diacetyl and glyoxal) in a sample of Iraqi patient with type 2 diabetic retinopathy

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Abstract
Diabetes mellitus is a common endocrine and metabolic diseases leading to impaired metabolism of carbohydrate, lipid, and protein. Hyperglycemia caused by complete or relative insulin deficiency, insulin defective secretion and incorrect action. Diabetic retinopathy is characterized by microvascular lesions such as impaired blood flow regulation, increased vasopermeability, capillary basement membrane thickening, microaneurysm formation, capillary dropout, and eventually widespread nonperfusion and ischemia. Dicarbonyl stress is a dysfunctional state where methylglyoxal (MGO) and other reactive alpha-oxaldehyde metabolites accumulate as consequence of their increased formation or decreased activity of the detoxifying systems. In diabetic patients, MGO and MGO-related advanced end products (AGEs) are responsible for many diabetes-related complications.

Keywords: Dicarbonyl compound, Diabetes Mellitus, HBA1c, Diabetic retinopathy

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Introduction
Type 2 Diabetes mellitus is a long-term metabolic disorder that is characterized by high blood sugar, insulin resistance, and relative lack of insulin.\textsuperscript{[1]} Common symptoms include increased thirst, frequent urination, and unexplained weight loss.\textsuperscript{[2]} Symptoms may also include increased hunger, feeling tired, and sores that do not heal.\textsuperscript{[3]} Often symptoms come on slowly.\textsuperscript{[4]} Long-term complications from high blood sugar include heart disease, strokes, diabetic retinopathy which can result in blindness, kidney failure, and poor blood flow in the limbs which may lead to amputations.\textsuperscript{[5]}

Type 2 diabetes is usually a chronic disease associated with a decade shorter life expectancy.\textsuperscript{[6,7]} This is partially due to a number of complications associated with it, including: two complications\textsuperscript{[8]} to four times the risk of cardiovascular disease, including ischemic heart disease and stroke; a 20-fold increase in lower limb amputations, and increased rates of hospitalizations.\textsuperscript{[9]} In the developed world, Type 2 diabetes, and increasingly elsewhere, is the leading cause of non-traumatic blindness and kidney failure.\textsuperscript{[10]} It has also been linked to increased risk of cognitive dysfunction and dementia through disease processes such as Alzheimer's disease and vascular dementia.\textsuperscript{[11]}

Diabetic retinopathy (DR) is the primary cause of visual impairment in the working-age population of the Western world.\textsuperscript{[12]} Among microvascular complications related to diabetes mellitus such as nephropathy and neuropathy, DR is the most common. The prevalence rate for DR for all adults with diabetes aged 40 and older is 28.5% in the
United States (4.2 million people) while estimated at 34.6% worldwide (93 million people) \[^{12,13}\]. With the prevalence of diabetes expected to continue to rise, the prevalence of DR in the United States by the year 2020 is expected to be 6 million persons with 1.34 million persons having vision-threatening disease \[^{14}\].

Dicarbonyl compounds (-DCs), a class of low molecular weights but highly reactive compounds, play an important role in food chemistry and biochemistry. In foods, they are the key precursors of color and aroma. However, they also have negative impacts on various foods since they can cause losses of nutrition through modifying proteins and thereby generating advanced glactation end products (AGEs) \[^{15}\]. Advanced glactation end products (AGEs) which accumulate under hyperglycemic conditions are thought to play an important role in the pathogenesis of diabetic retinopathy. AGEs arise primarily by the modification of amine groups of proteins by reactive dicarbonyls such as methylglyoxal \[^{16}\].

**Materials and method**

A total of 90 subject 30 Diabetic patients with complicated ritenopathy aged (30-65 years) attending Iben Al-haythem Eye hospital and 30 diabetic patients aged (30-65 years) without retinopathy attending Natienal diabetes center / Al-mustansryah university And 30 apperently healthy subject as control group, all these subject were enrolled in this study ,from May – Sep 2019.

**Sample collection**

Tow ml (2ml) put in EDTA tubes for HBA1c measuring and the remaining 5ml put into gel tube which allow to clot at room temp. For 20 mints and then centrifuged for 10 mints then the serum used for the measuring fasting blood glucose, lipid profile and blood urea, creatinine. The remaining serum was put in Eppendorf tube and stored at -20 °c for measuring of insulin.

**Method**

HOMA IR = fasting insulin *fasting glucose/405

Fasting insulin Mn/l

Fasting glucose mg /dl

**Estimation of Dicarbonyl by HPLC**

**Instrumentation**

A Gilson HPLC instrument was used, consisting of a 307 pump, a 115 UV variable wavelength detector, selected to 315 nm, an injection valve with a 20µL loop and a 25 cm x 4.6 mm, 5 µm particle size, reverse phase C18 column from Phase Separation Ltd. Results were registered in a Specific Data Jet integrator. Isocratic elution was used, with a flow rate of 0.8 mL/min. A mixture 80:20 acetonitrile /0.04 M acetate buffer pH=4.5 was used as eluent, after being degassed with a Schleicher & Schuell GV 050/0 vacuum filter holder, equipped with 0.2 µm S & S NL 16 membrane filters., Before injection in the HPLC column, samples were passed through a Gelman Acrodisc 13 CR PTFE 0.45 µm syringe filter. Solid phase extraction columns of 200 mg (3 mL) and 500 mg (6 mL) were Bond Elut from Varian and the elution was facilitated using a Vac Elute system.
Reagents and Solutions
Solutions of glyoxal, methylglyoxal, and diacetyl were prepared from the commercial products obtained from Aldrich. All other chemicals used were of analytical grade. Distilled water purified in a Millipore system was used for preparation of solutions. The o-phenylenediamine (OPDA) derivatization solution was prepared fresh before use by dissolution of the convenient amount of this compound (Merck) in the buffer solution used for derivatization. This solution should be kept in a dark place and handled carefully since o-phenylenediamine is toxic and may cause allergenic reactions. The samples of beer and of wine were directly purchased in a store.

Procedures
The procedure developed for the determination of the compounds was adapted from Verhagen et al.8 and is represented in Figure 2. After being 10 mL of water (B). As will be seen in the discussion of the results, these conditions (volume of sample and capacity of the extraction column SPE1) were optimized for beer. Less polar interferences are retained and the more polar α-diketones are collected (C), derivatized during 30 minutes with 5 mL of 0.5% ophenylenediamine (previously passed through another solid phase extraction column to remove interference impurities), and diluted with pH=4.5 acetate buffer to 25 mL in a volumetric flask. These 25 mL of reactant mixture (D) are passed through the extraction column SPE2 (200 mg of capacity), where the quinoxalines are retained (they are less polar than the α-dicarbonyl compounds GLYOXAL, METHYLGLYOXAL, AND DIACETYL from which they were formed) and o-phenylenediamine (more polar, as in acetate buffer it is protonated) is washed out almost completely with 2 mL acetate buffer (E). This buffer is removed by forcing air through the column with a syringe. Then, 1 mL of acetonitrile (F) is injected into the column to extract the quinoxalines; the extract is expelled from the column by forcing air again, and injected in the HPLC column, where the quinoxalines are separated using as eluent a solution of acetonitrile/aqueous acetate buffer (G) and detected spectrophotometrically at 315 nm.[15]

Results
Ninety subjects divided to three groups, 30 with diabetic retinopathy, 30 with type 2 diabetes mellitus, and 30 as controls. According to data analysis, no significant differences p≤0.05 was found between the studies groups regarding to their age according to table (1).
Table (1): A comparison between studies of groups in age

<table>
<thead>
<tr>
<th></th>
<th>Gender</th>
<th>No</th>
<th>Mean ±STD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Female/Age</td>
<td>11</td>
<td>45.36±4.29</td>
<td>0.1NS</td>
</tr>
<tr>
<td></td>
<td>Male/Age</td>
<td>19</td>
<td>46.57±5.25</td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>Female</td>
<td>12</td>
<td>48.41±4.25</td>
<td>0.1NS</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>18</td>
<td>47.88±6.74</td>
<td></td>
</tr>
<tr>
<td>Retino</td>
<td>Female</td>
<td>15</td>
<td>50.86±9.50</td>
<td>0.22NS</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>15</td>
<td>51.53±9.76</td>
<td></td>
</tr>
</tbody>
</table>

Means with a different letter in the same column significantly different (P≤0.05)
NS= not significant.

As shown in table (2,3,4) serum level of (glyoxal, methylglyoxal, and diacetyl, urea, HbA1c, TG, WC, Fasting glucose, fasting insulin) were significantly higher in retinopathy and type 2 diabetes mellitus compared to control, while no significant difference in body mass index and creatinine.

Table (2): mean±SD of serum urea, creatinine and dicarbonyl

<table>
<thead>
<tr>
<th></th>
<th>Urea</th>
<th>Creatinine</th>
<th>Glyoxal</th>
<th>Methylglyoxal</th>
<th>Diacetyl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14.30±3.63b</td>
<td>0.88±0.30a</td>
<td>0.055±0.004a</td>
<td>0.033±0.03a</td>
<td>0.35±0.03a</td>
</tr>
<tr>
<td>DM</td>
<td>34.50±10.79a</td>
<td>0.78±0.21a</td>
<td>0.068±0.09a</td>
<td>0.015±0.002b</td>
<td>0.27±0.02b</td>
</tr>
<tr>
<td>Retinopathy</td>
<td>33.36±10.94a</td>
<td>0.90±0.31a</td>
<td>0.017±0.004b</td>
<td>0.004±0.006c</td>
<td>0.22±0.01c</td>
</tr>
<tr>
<td>LSD</td>
<td>4.6812</td>
<td>0.1437</td>
<td>0.0276</td>
<td>0.0108</td>
<td>0.0132</td>
</tr>
<tr>
<td>p-value</td>
<td>0.001</td>
<td>0.1NS</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Table (3): mean±SD of serum insulin, glucose, Triglyceride, cholesterol, Glycated hemoglobin, insulin resistance

<table>
<thead>
<tr>
<th></th>
<th>Insulin</th>
<th>Glucose</th>
<th>TG</th>
<th>Cholesterol</th>
<th>HbA1c</th>
<th>IR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.34±2.01b</td>
<td>81.96±6.89c</td>
<td>99.90±19.67b</td>
<td>160.13±14.54b</td>
<td>5.14±0.38c</td>
<td>1.27±0.39c</td>
</tr>
<tr>
<td>DM</td>
<td>8.51±5.36b</td>
<td>211.06±73.83</td>
<td>217.66±108.03a</td>
<td>197.20±56.67a</td>
<td>8.71±1.84a</td>
<td>16.96±9.06b</td>
</tr>
<tr>
<td>Retinopathy</td>
<td>17.78±24.21a</td>
<td>252.23±86.02a</td>
<td>197.20±56.67a</td>
<td>217.66±108.13a</td>
<td>8.01±1.11b</td>
<td>24.89±22.32a</td>
</tr>
<tr>
<td>LSD</td>
<td>7.3736</td>
<td>33.564</td>
<td>36.64</td>
<td>36.429</td>
<td>0.6487</td>
<td>7.1397</td>
</tr>
<tr>
<td>p-value</td>
<td>0.006</td>
<td>0.001</td>
<td>0.001</td>
<td>0.008</td>
<td>0.001</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Table (4): mean±SD of serum Body mass index and waist circumference

<table>
<thead>
<tr>
<th></th>
<th>BMI</th>
<th>Waist C.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25.56±3.61b</td>
<td>83.53±15.37b</td>
</tr>
<tr>
<td>DM</td>
<td>29.50±4.45a</td>
<td>101.80±10.66a</td>
</tr>
<tr>
<td>Retinopathy</td>
<td>27.94±4.35a</td>
<td>90.16±15.56b</td>
</tr>
<tr>
<td>LSD</td>
<td>2.1339</td>
<td>7.2118</td>
</tr>
<tr>
<td>p-value</td>
<td>0.7NS</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Discussion
MGO is the most reactive dicarbonyl and of the highest endogenous flux (ca. 3 mmol per day). Therefore, it is commonly the primary concern. Glyoxal is definitely an interesting issue in the context of carbonyl stress and its
physiological significance in diabetes. In addition to Maillard reaction, glyoxal is a side product of lipid peroxidation. It is important as a glycation agent in physiological systems and a precursor of AGEs. A specific glyoxal-derived AGE is a lysine-lysine crosslinking structure named glyoxal-lysine dimer or GOLD. Investigators have reported an age-related increase of GOLD in human lens proteins as well as in diabetic state \[18, 19, 20, \text{and} 21\].

The rapid reaction of glyoxal with proteins may account for the difficulty in its measurement in biological samples \[22\]. However, there are reports on significant increases of plasma glyoxal concentrations in uremic \[23\] and diabetic patients \[24\]. Methylglyoxal (MGO) and dicarbonyl stress are widely accepted as pathogenesis factors in diabetes and its related complications. Increasing evidence implicates MGO in other disease models \[25\]. We already know that diabetes affects a large number of diverse individuals, who have similar disease courses and complications. Methylglyoxal is up to 20,000-fold more reactive than glucose in glycation processes \[26\].

In diabetic patients, MGO and MGO-related advanced end products (AGEs) are responsible for many diabetes-related complications \[27\]. Moreover, diabetic nephropathy is associated with MGO-related AGEs rather than HbA1C, which reflects the long-term blood sugar level \[28\]. High MGO levels are found in diabetic patients, and are thought to be due to excess blood sugar \[25\]. Methylglyoxal is formed through non-oxidative mechanisms from triose phosphates during anaerobic glycolysis. This metabolite can modify amino acids, nucleic acids, and proteins (Rabbani & Thornalley, 2015). Methylglyoxal reacts with arginine, lysine, and cysteine residues of proteins to form AGEs \[17\]. Methylglyoxal and its downstream products, AGEs, are together called dicarbonyl stressors. They are well-known contributors to the development of diabetic complications. Recently, dicarbonyl stress has been established as a pathogenesis factor in diseases other than diabetes, such as renal failure \[29\], hypertension, and sepsis. These diseases present normal blood sugar, yet they share some features with diabetes. A feature common to these carbonyl-stress conditions is systemic damage causing disease progression and complications. Another study focusing on type II DM also reveals serum MGO level is associated with DMN \[30\]. Compared to the traditional diabetic marker hemoglobin A1C (HbA1C), skin AGEs such as MG-H1 correlate better with diabetic nephropathy parameters \[31\]. Another study shows that urine d-lactate, a downstream metabolite of MGO, is higher in DMN subjects than healthy subjects \[29\]. These clinical data suggest MGO is crucial for progression of diabetic nephropathy \[32\].

The primary biosynthetic pathway of MG in diabetic patients remains elusive, but MG is known to be produced from a variety of sources. That is, MG can be produced not only from glucose but also from a variety of substances and is not necessarily produced from hyperglycemia only \[33\]. Elevated blood concentrations of MG have been reported in type 2 diabetics \[34\], and it has been reported that plasma free MG-derived hydroimidazolone was higher in the type 1 diabetics as compared with the nondiabetics \[35\]. It is considered that monitoring of MG biogenesis in diabetic patients might help to assess the risk of progression of diabetic complications. Under physiological conditions, MG exists in two forms, i.e. as a free dicarbonyl compound (unbound form) and bound to protein residues, known as MG-derivative AGEs. MG in the free form is very unstable, has short biological survival, and sophisticated techniques are required for its quantitative evaluation. Our results of free MG measurement have shown excessive production in diabetic state, detected in both plasma and whole blood samples \[36\]. We found MG overproduction to correlate tightly with glycemia fluctuation.

**Conclusions**

A highly significant decrease in the levels of alpha dicarbonyl (glyoxal & methylglyoxal), while significant increase diacetyle were shown in both (DM & DR) patients in comparison with their levels in the control group.

**References**

22. Nakajima K, Ohta K, Mostefaoui TA, Chai W, Utsukihara T, Horiuchi CA, Murakami M. Glyoxal sample preparation for high-performance liquid chromatographic detection of 2, 4-dinitro-phenylhydrazone...
derivative: suppression of polymerization and mono-derivative formation by using methanol medium.


