Evaluation the Oxidative Stress in Patients with Diabetes Mellitus (Type I and Type II) and Study Genetic Level of Glutathione-S-transferase mu 1.

Nisreen J. Mohammed, Moaed Emran AL-Gazally and Maysam Ali Ameen Awadh

Abstract
Diabetes mellitus is a group of metabolic diseases. Hyperglycemia in diabetes creates free radicals. These free radicals produce oxidative stress and thus debilitate the endogenous antioxidant defense system. The present study was conducted in (75) diabetic patients, (25) of them were treated with insulin, other (25) were using insulin and metformin and the last (25) were on metformin and glibinclimide. The study also included (25) apparently healthy subjects were taken as control group. These groups were collected from Merjan Teaching Hospital from November 2013 to April 2014. Both the (insulin & metformin) and (metformin & glibinclimide groups) groups were highly significant decrease in the total antioxidant when compared with control group (p ≤0.01) and no significant difference between them also the results showed insulin group was significant decrease against control (p value = 0.036). LSD test shows there was no significant difference in glutathione S-transferase activity in three treated groups (p ≥ 0.05). In the gene level of the study, genotyping of glutathione S-transferase mu 1 gene by PCR were defined as GSTM1 and GSTM0 or deletion association to the present and absence of the guanine nucleotide in the gene sequence. There was statistically difference in the genotyping distribution and the frequency of GSTM0 among study groups were 44% for insulin and metformin group, 68% for metformin and glibinclimide, 44% insulin and 28% for control healthy group. Conclusions raised from the present study involve that diabetes mellitus association with decrease total anti-oxidant capacity. Genetic polymorphism of glutathione S-transferase mu 1 gene may be considered as risk factor for both types of diabetes mellitus.

Introductions:
Antioxidant is any substance that when present at low concentration compared with that of an oxidizable substrate, significantly delays or inhibit oxidation of substrate. This includes compound of non-enzymatic as walls an enzymatic nature (Halliwell 2001).
Glutathione S-transferase (GST) is a family of enzymes that plays an important role in detoxification of xenobiotics. GST catalyzes attachment of the thiol of glutathione to electrophiles. Glutathione is used to scavenge potentially toxic compounds including those produced as a result of oxidative stress and is part of the defense mechanism neutralizing the mutagenic, carcinogenic and toxic effects of such compounds (Udomsinprasert, et al; 2005). Cytosolic GSTs are classified into 13 classes according to their structures: alpha, beta, delta, epsilon, zeta, theta, mu, nu, pi, sigma, tau, phi, and omega. (Oakley 2011).
A physical map has been developed of the human class Mu GST genes on chromosome 1p13.3. The GST genes in this group are dispersed arowned 20 kilo base pairs (kb) separator, and orchestrated as 5*-GSTM4–GSTM2–GSTM1–GSTM5-3*. This map has been utilized to confine the end purposes of the polymorphic GSTM1 cancellation (Shi-jie, et al; 1998).

Material and methods:
The diabetic gropes who subjected to this study were divided into (3) gropes each group had (25) patients: First group had treated with insulin, their age ranging from (14-77) years, second group had treated with insulin companied with metformin, their ages ranging from (18-64) years, third group would treated with combination of oral hypoglycemic drugs metformin and glibinclimide. All the patients had suffered from DM for about 5-15 years, they should be not smokers or pregnant, they had no other chronic disease like hypertension and they had taken medications of DM regularly.

1. Total antioxidant concentrations:
Total Anti-Oxidant Concentration determined by detecting a broad absorbance peak around 570 nm of chelating the reduced Cu+ to colorimetric probe and these absorbance proportional to the TAC according to Bio Vision improved the TAC Assay Kit.

2. Glutathione S-transferase Activity:
The conjugation of L-glutathione to (1 – chloro 2 , 4 – Dinitro benzene) CDNB is catalyzed by GST through the thiol group of the glutathione (Habig, et al.; 1974).

3. Gene Level:
In this experiment, Primers were designed based on the cDNA sequence for human GSTµ. The primers hybridize
to the 5’ region of exon 4 (5’-CTGCCCTACTTGATTGATGGG-3’) and the 3’ region of exon 5 (5’-CTGGATTGTAGCAGATCATGC-3’) of GSTµ1 (Seidegard, et al.; 1988).

Poly chain reactions had been occurred by mixing 12.5µl of master mix GoTaq® Green Master Mix (Promega Green Master Mix), 2µl of primer mix, 4µl of DNA extraction (200 ng) and 6.5µl of d.d.H2O. Net volume is 25µl. Primer mix consisted of 10µl of fir st primer and 10µl of second primer and complete volume to 100µl with d.d.H2O. Reactions were heated for 2 min at 94°C, 1 min at 55°C and 1.5 min at 72°C for 35 cycles in a Thermal Cycler. PCR products 273 bp were electrophoresed on an 8% acryl amide gel.

Results and discussions:

1. Total antioxidant concentrations:
The outcomes show that there was significant decrease in TAO in diabetic groups in contrast with control group and this may be due to increase serum glucose level result in increasing O\(^{-2}\) generation and this plays an important role in the formation of other ROS in DM and that causes increase oxidative stress against TAO (Nishikawa, et al., 2000).

Table (1) Mean difference of TAO in the study groups

<table>
<thead>
<tr>
<th>Variable</th>
<th>Groups</th>
<th>Mean ± SD</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>TAO concentration</td>
<td>Control</td>
<td>2.15± 0.38 mmolar</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Insulin &amp; Metformin</td>
<td>0.99± 0.58 mmolar</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td></td>
<td>Metformin&amp;Glibinclimide</td>
<td>0.98± 0.45 mmolar</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Insulin</td>
<td>1.56± 1.75 mmolar</td>
<td></td>
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</table>

Insulin & Metformin group and Metformin & Glibinclimide were decrease in TAO highly significantly (p value≤0.01) may be as a result of metformin side effect in reduced B12 absorption (BNF, 2007). Deficiency of B 12 is affecting on endogenous antioxidant defense system and this lead to decreased TAO (Wolfgang, et al.; 2001) and that is agree with the opinion of Schroeksnadel and his group who show that Vitamin B12 acts as enzyme cofactor. Low availability it leads to impaired remethylation of homocysteine to methionine and thus to homocysteine accumulation, as it is essential cofactor in homocysteine-methionine metabolism Recent data indicate that homocysteine accumulates secondarily due to heightened oxidative stress associated with immune activation (Schroecksnadel, et al.; 2010).

Insulin group had the less mean difference with control group (p=0.036) that means the less decreased in TAO among DM groups may be since insulin group was younger and TAO increase in young and decrease with ageing and that is the similar to Thavanati, et al; 2008 that found ageing decrease TAO and increase DNA damage by ROS.

Insulin resistance characterized by hyperinsulinemia is frequent in the majority of the individuals with type 2 diabetic mellitus. Insulin induces the release of H\(_2\)O\(_2\) when activating its receptors (Wiernsperger, 2003). Even though H\(_2\)O\(_2\) is a non-free radical; it is membrane permeable and can diffuse to other sites, different from its site of production. Chronic hyperinsulinemia together with the impaired antioxidant defenses in diabetes will lead to inefficient scavenging of H\(_2\)O\(_2\). In the presence of transition metals such as copper and iron, H\(_2\)O\(_2\) undergoes other reactions to generate •OH (Halliwell and Gutteridge, 2007).

2. Glutathione S-transeferase:
The statistical data showed significant decrease in GST activity in treated groups with respect with control group because of alteration of enzymatic activity of GST in hyperglycemia or change in enzyme gene expression (Bojunga et al., 2004) or a post translational of enzyme modification (glycation), or a combination thereof. It has been suggested that glycation of antioxidant enzymes due to hyperglycemia may be responsible for the reduction in GST activity (Wolff et al., 1991).

Table (2) Mean difference of GST activity in the study groups

<table>
<thead>
<tr>
<th>Variable</th>
<th>Groups</th>
<th>Mean ± SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST</td>
<td>Control</td>
<td>1.40± 0.71 U/L</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Insulin &amp; Metformin</td>
<td>0.36±0.34 U/L</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>GST</td>
<td>Metformin&amp;Glibinclimide</td>
<td>0.27± 0.07 U/L</td>
<td></td>
</tr>
<tr>
<td>GST</td>
<td>insulin</td>
<td>0.35± 0.59 U/L</td>
<td></td>
</tr>
</tbody>
</table>

*p value ≤ 0.05 is significant.

By using LSD test to compare between three treated groups there was no significant difference and the p value was ≥ 0.05.

Excessive oxidative stress due to diabetes mellitus, resulted in the down regulation of the expression GST-Mu gene (Sadi, et al; 2008). The decrease in the mRNA expressions of GST-Mu also reflected in both the protein expressions and the activities, indicating the transcriptional regulation of the gene. The reduction in the
mRNA of GST-Mu isoenzyme in diabetes could be due to oxidation of transcriptional factors responsible for the initiation machinery of antioxidant enzyme transcription process found in antioxidant response elements. The decrease could be also due to the decrease in the half lives of mRNAs since increased oxidative stress may lead to destabilization of mRNA (Navarro, et al; 2008) and this explain the cause of there was no significant difference in GST activity and HbA1c between three treated group.

The studies show that increased oxidative stress in presence of hyperglycemia may lead to increased availability of transition metals like copper released from its storage site. Copper in its free form is a potent cytotoxic element because of its redox chemistry it readily participates in generation ROS. This elevated ROS could have consumed considerable amount of total thiol, which may explain the decreased level of total thiols in patient with hyperglycemia. Previous studies on animal model suggest that, a combination of diabetes and copper deficiency resulted in increased level of hepatic GST (Sarkar, et al; 2010).

3. Genomic level:
Genotyping of the GSTM1 gene is GSTM1*A/A, GSTM1*B/B or GSTM1*A/B genotypes. But due to presence of a deletion in this gene, the possible genotypes would be GSTM1*A/A, GSTM1*A/0, GSTM1*B/B, GSTM1*B/0, GSTM1*A/B or GSTM1*0/0. PCR readings cannot differentiate between GSTM1*A/A, GSTM1*A/0 genotype, as both appear as positive band at the specific locus, are considered to be GSTM1*A wild genotype, while GSTM1*B/B and GSTM1*B/0 are considered as GSTM1*B mutant genotype (Alshagga, et al; 2011).

In this study the result showed that, the differentiation between GSTM1*A/A, GSTM1*A/0, GSTM1*B/B, GSTM1*B/0, GSTM1*A/B (represent positive results) in the hand and GSTM1*0/0(negative results) which called null or deletions by PCR.

Percentage of control group was 28% in Babylon community and this agree with Seidgard, et al; 1988 who showed that total of 20 to 50% of persons had gene deletion, is called the GSTM1*0, or null allele (Seidgard et al., 1988). Frequencies of GSTM1 homozygous null genotype (GSTM1*0) were 42%-60% in Caucasians and 16%-36% in Africans (Garte, et al; 2001).

Figure shows the product of PCR in polyacrylamide gel 8% under UV light (1, 3,4,5,7,8,10 and 11) are normal GSTµ1. (2, 9, and 12) are deleted GSTµ1 6 is 100 b.p. DNA ladder.

In this study, result shows that the largest percentage of deletion occur in metformin& glibinclimide group 68% who represented patients with type II diabetes mellitus that mean GSTM1*0 represent one of risk factor for type II diabetes mellitus and that is reaching agreement with Bhandari who proved that the GSTT1 and GSTM1 genes, alone or combined, have an influence on the risk of having type 2 diabetes mellitus (Bhandari 2014) and Bid, et al ;who proved that the association of a combined effect of GSTM1, T1 and P1 genotypes in a representative cohort of Indian patients with T2DM. Since significant association was seen in GSTM1 null and GSTP1 and multiple association in GSTM1 null, T1 present and P1 (I/I), these polymorphisms can be screened in the population to determine the diabetic risk (Bid, et al; 2010).

The result of this theses is disagree with Guoying, et al. who show that There was no association between either GSTM1 polymorphism and risk of T2 DM (Guoying, et al;2006).

The test displays that GSTM1*0 in insulin and insulin & metformin groups was the same 44% which were significant increase than present of control group and these groups represent type I DM that mean GSTM1*0 represent as risk factor for type I DM and that is agree with the result of Lynn, et al; who suggest that
the GSTM1 null genotype is associated with T1D protection and T1D age-at-onset and that susceptibility to T1D may involve GST conjugation (Lynn, et al; 2005), similarly Vojtková, et al; who state that genotype combination GST T1 null/M1 wild was significantly more prevalent in subjects with diabetes and represented 2.9-fold risk for T1D developing (Vojtková, et al; 2013).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (%)</th>
<th>Insulin &amp; Metformin (%)</th>
<th>Metformin &amp; Glibinclimide (%)</th>
<th>Insulin (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTM1 Deletion</td>
<td>7 (28.0)</td>
<td>11 (44.0)</td>
<td>17 (68.0)</td>
<td>11 (44.0)</td>
<td>0.042*</td>
</tr>
<tr>
<td>Normal</td>
<td>18 (72.0)</td>
<td>14 (56.0)</td>
<td>8 (32.0)</td>
<td>14 (56.0)</td>
<td></td>
</tr>
</tbody>
</table>

*p value ≤ 0.05 is significant.

Deletion in the GSTM1 gene had a protective role for diabetic retinopathy. A large number of studies on GSTM1-0 and/or GSTT1-0 null genotypes report an increased risk for development and progression of rheumatoid arthritis and asthma (Tamer, et al; 2004).

A large-scale cohort study in Egyptian population may confirm the role of GSTM1, T1 and P1 gene polymorphisms in the pathogenesis of T2DM and its related complications (Zaki, et al; 2014).

Significant correlation between GSTM1 null genotype and retinopathy could indicate this fact that impairs cellular metabolism result in increased free radicals and oxidative pressure. Therefore, GST null genotypes may result in decrease antioxidant capacity which causes side effects of diabetes (Dadbinpour, et al; 2013).

References:
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