Assessment of Oxidative Stress and Homocysteine Level in Patients with and without Type 2 Diabetic Retinopathy

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Article information	Abstract
  *Corresponding author at: Department of Clinical Biochemistry, Mashhad university of Medical Science, Mashhad, Iran E-mail: Hafezheydari@ymail.com	**Background**: Oxidative stress and mild hyperhomocysteinemia are independent risk factors of vascular diseases and present in type 2 diabetes. Our aim in this study is to investigate role of hyperhomocysteinemia, oxidative stress and association of homocysteine level with oxidative stress in the development of diabetic retinopathy. **Materials and Methods:** Forty type 2 diabetic patients with retinopathy and sixty without retinopathy subjects were included to the study. Plasma homocysteine level, prooxidant-antioxidant balance and HbA1c concentration was measured in two groups, also we tested 50 healthy volunteers as control. **Results**: HbA1c concentration in patients is significantly higher than healthy subject and positive correlation was found between HbA1c and retinopathy in diabetes patients. Plasma levels of homocysteine are significantly higher in diabetic patients compared to healthy individuals. However, there is no significant differentiate in homocysteine plasma levels in patients with and without retinopathy. Oxidative stress is higher in diabetic patient compared to healthy subjects; and also there is significant association between retinopathy and oxidative stress. Our data don't show any significant correlation between HbA1c and homocysteine levels and homocysteine level with oxidative stress, however there is positive correlation between oxidative stress and HbA1c concentration. **Conclusion**: Our findings confirm elevation of homocysteine in diabetic patients; however there isn't significant correlation between homocysteine levels and presence of diabetic retinopathy in type 2 diabetic patients. In agreement with previous data oxidative stress significantly associated with development of retinopathy.

Introduction

Retinopathy is one of most specific complication of diabetes and eventually leads to blindness [1, 2]. Approximately 5% of the global prevalence of blindness is considered to be due to diabetic retinopathy [3, 4]. Hence it is extremely important to characterize factors affect development of retinopathy. Numerous factors such as hypertension [5, 6], plasma glucose level, Glycated hemoglobin (HbA1c), nutritional status influence on development and progression of diabetic retinopathy [7-11]. Further studies are essential to identify involvement of other factors and investigate association of discovered risk factors.

Mild hyperhomocysteinemia and oxidative stress are associated with premature vascular disease [12, 13]. Oxidative stress is imbalance of oxygen free radicals and biological system antioxidant capacities to detoxify reactive intermediate metabolites or repairing of induced damages [14]. Cellular constitutes continuously exposed to oxidative stress in course of life time. Reactive oxygen species can damage to lipids, DNA, proteins of endothelial cells, altering endothelial structure and function [15]. Oxidative stress is suspected important in pathogenesis of many various viscular diseases [16]. Homocysteine (Hcy) is an amino acid containing thiol produced from methionine metabolism [17]. Mild hyperhomocysteinemia is defined as plasma homocysteine level in excess of 15 µmol/liter [18]. Mild hyperhomocysteinemia alter endothelial function through induction of smooth cell proliferation, reduction nitric oxide (NO) synthesis, induction of oxidative stress and inhibition of endothelial cell arginine transport. However, the roles of mild hyperhomocysteinemia, oxidative stress and correlation of homocysteine with oxidative stress in the development of retinopathy in diabetic patients are still unknown and require further investigation.

Because a relationship may be hypothesized between enhancement of homocysteine and induction of oxidative stress in diabetes patient with development of retinopathy, the aim of this study was to investigate the mean serum level of homocysteine and oxidative stress in type 2 diabetes mellitus patients with retinopathy and compared to age and sex matched patients without retinopathy and healthy case controls. Also we try to determine association of homocysteine and oxidative stress in genesis of retinopathy in diabetic patients.

Materials and Methods

Study participants: The research protocol was approved by ethical committee of medical science university of Mashhad. One hundred patients with type 2

diabetes (44 male and 56 women, mean age, 45.9±6.5 years) and 50 healthy subjects (20 male and 30 women, mean age, 42±5.8) were studied. The final patient group includes 40 patients with retinopathy and 60 patients without retinopathy. A detailed medical history was obtained from each patient. All patients who participated in the study referred by endocrinologist and were treated with oral metformin. Exclusion criteria's were: drugs affecting homocysteine metabolism, smoking] or alcohol consumption, thyroid disease, vitamin B_6 and B_{12} consumption and renal diseases [20-24].

A control group of 50 subjects was recruited from a large population of healthy volunteers. The patients and control groups matched for sex, age, and smoking and alcohol consumption habits. Peripheral blood samples was collected in tubes containing EDTA as anticoagulant at 8.00 a.m. after an overnight fasting and centrifuged within 30 min at 1500 g for 15 min at 4ºC. Plasma were separated and stored at -70ºC until analyzed. Total glycated hemoglobin determined by affinity chromatography. Plasma homocysteine level and oxidant-antioxidant balance was measured for diabetic patients and healthy subjects.

Laboratory analysis Homocysteine (Hcy) assay: The plasma homocysteine level was analyzed by enzymatic immunoassay method (EIA) by Axis-Shield Homocysteine Enzyme Immunoassay (EIA) kits. Briefly, in this method, protein-bound Hcy is reduced to free Hcy and then reacts with serine catalyzed by cystathionine beta synthase to form L-cystathionine. Cystathionin is cleavaged to homocysteine, pyruvate and ammonia by cystathionine beta lyase. Pyruvate is converted to lactate with NADH as coenzyme by lactate dehydrogenase. Concentration of homocysteine is directly proportional to the rate of NADH conversion to NAD. Quantification limit of this assay (CV < 20%) is 1.0 µmol/L.

Prooxidant-antioxidant balance assay (PAB assay): For assessment of oxidative stress we use PAB assay method. Briefly, five standard solutions (0, 25%, 50%, 75% and 100%) were prepared by mixing various proportions of 250 µM hydrogen peroxide with 3 mM uric acid in 10 mM NaOH. For preparation of TMB cation, 60 mg TMB powder dissolved in 10 ml DMSO, then 20 ml of acetate buffer (0.05 M buffer, pH=4.5) was dissolved in 400 µl of TMB/DMSO. Chloramines T dissolved in distilled water and 100 mM solution prepared. This solution must prepare freshly.

Seventy microliters of chloramine T added to 20 ml, mixed well and incubated at room temperature in dark place for 2 hr. Solution of peroxidase enzyme (25 units) was added to 20 ml TMB cation, aliquated in 1 ml and stored at -20ºC. For preparing TMB solution, 200 µl of TMB/DMSO was added into 10 ml of acetate buffer (0.05 M buffer, pH 5.8); the working solution was prepared by mixing 1 ml TMB cation with 10 ml of TMB solution, incubated for 2 min at room temperature in a dark place and was immediately used. Ten microliters of all samples, standard and blank solutions (distilled water) added to each well of a 96 well plate, next 200 µl of working solution added to each well and incubated in a dark place

at 37ºC for 12 min. At the end of 12 min, 100 µl of 2N HCl was added to each well to terminate reaction and absorbance value measured by ELISA reader at 450 nm with a reference wavelength of 620 or 570 nm. Using standard values standard curve provided and value of unknown samples were calculated from standard curve. HK is arbitrary unit for expressing of PAB values. HK indicate percentage of hydrogen peroxide in standard solutions.

Statistical analysis: Data were analyzed using a SPSS-14 program .The student *t*-test was used to test the significance of differences between the two means. The Mann-Whitney *U* test was used to compare the means of more than two independent groups. The correlation coefficient and the Chi-squared tests were used to measure the relationship between two quantitative and qualitative variables respectively. Dubin-Watson test were used for regression analysis. Differences were considered significant at $p<0.05$. All results are reported as mean±SD.

Results

Homocysteine levels were significantly higher in diabetic patients as whole compared to controls (mean 27.1±2.2 vs. 15.4±5.4 µmol/L, $p<0.001$). Oxidant-antioxidant balance was twice higher in diabetic patients as in the controls (54.8±7.2 vs. 20.2±9.2 HK, $p<0.0001$).

The tested variables in the subgroups with retinopathy and without retinopathy are compared in figure 2 and 3. Plasma homocysteine was not statistically significant difference in diabetic patients with and without retinopathy. Both subgroups included the same of hyperhomocysteinemia (means 27.7±2.2 vs. 26.7±3.3 µmol/L in patients with and without retinopathy respectively).

Oxidant-antioxidant balance in diabetes patients with retinopathy differ significantly from diabetes patients without retinopathy (mean 76.8±7.1 vs 32.9±4.3 HK, $p=0.001$). Regression analysis revealed no correlation among Hcy and oxidant-antioxidant balance regardless of the way the variables were considered (r=0.100).

Discussion

Although many studies have evaluated the association between homocysteine and diabetic retinopathy, the results are varied and inconsistent [25]. Various studies have proposed that mild hyperhomocysteinemia and oxidative stress may play a role in development of diabetic retinopathy [26, 27]. In contrast, some studies have suggested that there is no significant correlation between homocysteine and diabetic retinopathy [28].

Previous studies suggest high level of homocysteine induce oxidative stress via thiolactone formation and disrupt endothelium integrity [29]. Therefore, we design this study to evaluate possible role of total plasma homocysteine level and oxidative stress in genesis of diabetic retinopathy.
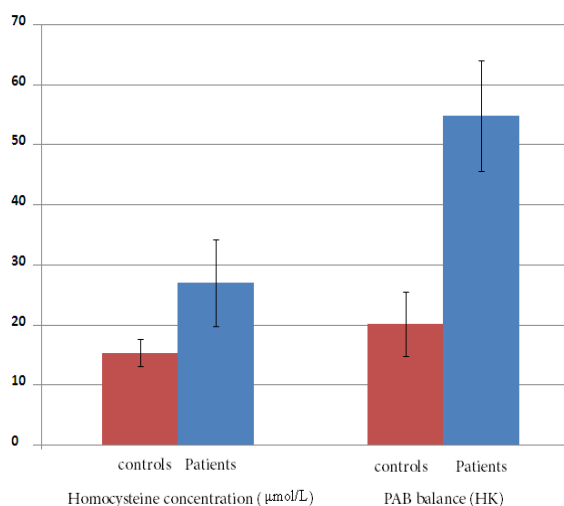
Figure 1. Plasma homocysteine concentration and oxidant-antioxidant balance in diabetic patients and controls
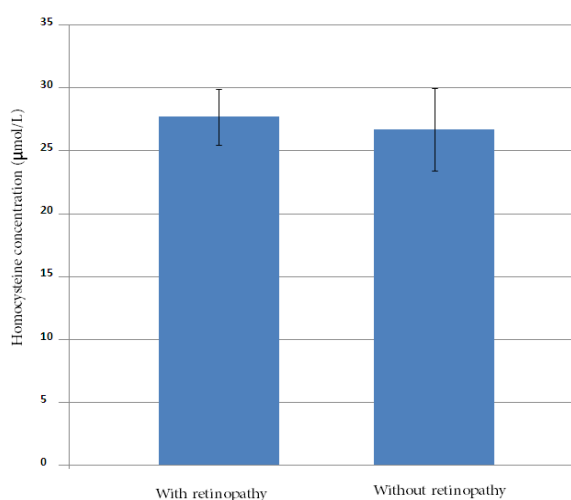

Figure 2. Plasma homocysteine level in diabetic patients with and without retinopathy
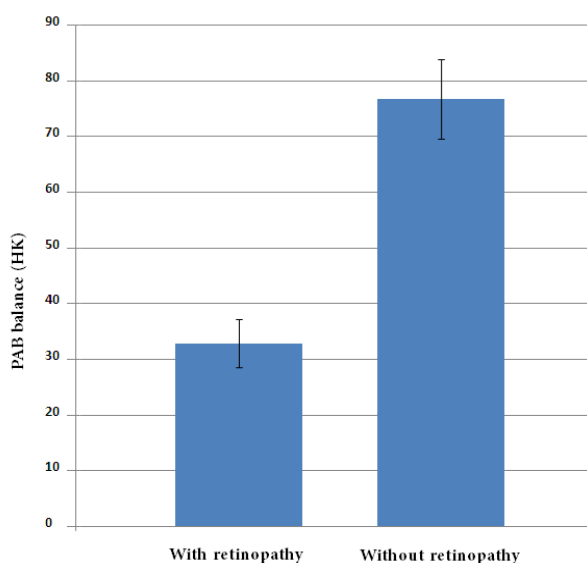

Figure 3. Prooxidant-antioxidant balance in diabetic patients with and without retinopathy

We asses total plasma homocysteine concentration and use oxidative-antioxidant balance as an index of oxidative stress. In our study, HbA1c, prooxidant-antioxidant balance and homocysteine concentration determined simultaneously in collected samples, hence relation of homocysteine, HbA1c and oxidative stress could be precisely determined.

In agreement with previous study [25], the results of the present study show that plasma homocysteine level is significantly higher in diabetic patients as a whole and in retinopathic and non-retinopathic subgroups considered separately than in the controls. These results confirm presence of hyperhomocysteinemia in diabetic patient, as reported by some of previous studies [30, 31]. Although homocysteine level in diabetic patients is higher than controls but there is no significant differentiate between two subgroups. Thus, based on our results, there isn't an important relationship between homocysteine and diabetic retinopathy. Our findings agreed with some of previous studies [32, 33].

In agreement with previous study, involvement of oxidative stress in diabetes is confirmed by significantly higher oxidant-antioxidant balance in diabetic compared to controls. We found oxidant-antioxidant balance is higher in retinopathic group and non-retinopathic subgroups compared to controls. HbA1c concentration in subgroup with retinopathy is significantly higher than other subgroup [34, 35]. On base of previous studies, HbA1c concentration is index of glucose concentration and high levels of HbA1c indicate higher levels of glucose [36]. Hyperglycemia induces oxidative stress [37], hence high concentration of HbA1c and presence of oxidative stress in retinopathic patient confirm each other. Also there is significant correlation between HbA1c and oxidative stress with retinopathy that confirm previous result.

Regarding the relationship between Hcy and oxidative stress, some of previous studies report hyperhomocysteinemia induce oxidative stress [38, 39]. However according to our result homocysteine concentration has not significant difference in two subgroups, but oxidant-antioxidant levels is significantly associated with retinopathy and higher in patients with retinopathy than patients without retinopathy. Thus it appear in diabetic patient high levels of homocysteine is not entirely responsible for oxidative stress and probably hyperglycemia have most important than hyper-homocysteinemia. Presence of significance correlation between HbA1c concentration and retinopathy confirm this result.

In conclusion, our observations in agreement with previous studies support a role for hyperglycemia and oxidative stress in diabetic retinopathy. However, according to our result homocysteine is not associated with the presence of diabetic retinopathy in type 2 diabetic patients [40, 41].

Finally, our study has several limitations. Although we controlled for retinopathy risk factors, we were not assessed some of factors linked to homocysteine such as

renal disease, red cell folate levels, genetic factors, depression and life style habits [42].

Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

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