

## **Study on the mechanism involving homocysteine-triggered vascular endothelial injury in diabetes.**

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### **Abstract**

**Background:** The study was conducted to investigate the primary mechanism via which plasma homocysteine (HCY) causes vascular endothelial injury (VEI) in patients with diabetes.

**Methods:** 286 patients with diabetes were assigned to two groups based on whether B ultrasound indicated plaque in the carotid artery. The parameters including blood HCY, and interleukin-8 (IL-8) were determined. The statistical differences in blood HCY, and IL-8 were determined by comparing the patients with carotid plaque to those without. Whether blood HCY was correlated with IL-8, and carotid artery ultrasound was determined.

**Results:** The patients with carotid plaque also had significantly elevated levels of plasma HCY and IL-8 when compared to those without carotid plaque, reaching up to  $17.58 \pm 5.71$  u mol/L ( $p < 0.001$ ) and  $79.69 \pm 24.85$  pg/ml ( $p < 0.001$ ), respectively. In two groups with diabetes, those with plaque had significantly thickened tunica media of right common carotid artery (RCCA) and increased resistance index of bilateral CCAs than those without plaque.

**Conclusions:** HCY can aggregate the VEI in patients with diabetes, and cause thickened tunica media of right common carotid artery (RCCA) and increased resistance index of bilateral CCAs. HCY may trigger VEI in patients with diabetes through effects of the cytokines such as IL-8.

**Keywords:** Homocysteine, Diabetes, Vascular endothelial injury.

*Accepted on January 11, 2017*

### **Introduction**

Diabetes Mellitus (DM) is a comprehensive metabolic disorder, which is characterized by hyperglycemia. The Vascular Complications (VC) of DM is main cause of disability and death. Diabetic vascular disorders (DVD) can be classified into two types, diabetic microangiopathy (DMIP) and diabetic macroangiopathy (DMAP).

Atherosclerosis is primary pathological change of DMAP, which increases the risk of myocardial infarction, stroke, intermittent claudication and ischemic gangrene. Currently, most reports suggested that high level of blood HCY was an independent risk factor for heart, brain, and peripheral vascular disorders.

There are studies in China that have investigated the correlation of plasma HCY level and incidence of diabetic vascular complications or severity. However, the relationship between HCY and diabetic VCs as well as mechanism remains largely unknown and requires further study. Therefore, the study of the role HCY plays in the development of diabetic VCs can render important theoretical and practical value.

### **Materials and Methods**

#### ***Selection of subjects***

286 patients with diabetes who were admitted to the endocrinology, cardiology and geriatrics departments between May 2015 and February 2016 were selected. The subjects should meet the following inclusion criteria:

1. Patients were diagnosed with diabetes according to the diagnostic criteria for diabetes. There were 151 male cases with mean age of 59.06 and 135 female cases with mean age of 61.44. The blood glucose was under relatively stable control. The acute complications such as High blood glucose toxicity, hypoglycemic coma, acute infections, diabetes ketosis and diabetic high permeability were excluded. All patients in this cohort were excluded for hypertension based on the systolic pressure less than 140 mmHg and the diastolic pressure less than 90 mmHg.
2. 57 cases were diagnosed with hypertension according to diagnostic criteria for hypertension, including 29 male cases with mean age of 66.34 and 28 female cases with mean age of 62.36. The secondary hypertension and abnormal glucose

metabolism (including diabetes, impaired fasting glucose and abnormal glucose tolerance) were excluded.

3. Medical history and laboratory test did not indicate disorders of blood system. Patients with liver and kidney disorders were excluded. The liver and kidney functions indicated by routine admission examination should be within normal range.

4. The patients did not have significant electrolyte disturbance, infection, immune system disorder and history of long-term use of hormones.

5. Patients with malignant tumors were excluded.

The patients with diabetes were assigned to two groups with or without carotid plaque. The following clinical data were collected. For patients with hypertension, only those with carotid patients were collected for clinical data.

### **Measurement of general conditions**

The patients were recorded for ages and genders. The morning rest lying blood pressures were determined. The mean of 3 measurements was recorded. The height and weight were measured. The body mass index (BMI ( $\text{kg}/\text{m}^2$ )= $\text{weight}/\text{height}^2$ ) was calculated. The waist circumference was determined. Information about smoking, drinking, medication and special life and work conditions were collected.

### **Determination and sample collection**

All subjects discontinued taking lipid control drug three days prior to blood collection or never took lipid control drug. High lipid diet and glucose-containing liquid should be avoided within three days. 8 ml of blood was collected via elbow vein on rest lying position in the morning after fasting for 12 h.

Venous blood (3 ml) was separated for serum (without anticoagulation). The Beckman 2700 analyzer (The kit was provided by Sichuan Maccura Biotechnology Co., Ltd.) was used to determine serum HCY.

Venous blood (3 ml) was anticoagulated by potassium oxalate/sodium fluoride, and analyzed by Beckman AU5800 automatic biochemical analyzer (The kit was provided by Beckman Coulter, American.) to determine Fasting Blood Glucose (FBG) using hexokinase method. The automatic glycated hemoglobin (GHb) analyzer (The kit was provided by TOSOH, Japan.) was used to determine GHb using high performance liquid chromatography.

Coagulant serum sample (5 ml) was analyzed by IMMULITE 1000 systems chemiluminescence immunity analyzer (The kit and calibration solution were provided by SIEMENS, Germany) to determine IL-8 using radioimmunoassay.

Two hours after breakfast, 2 mL of blood was collected via elbow vein and anticoagulated by potassium oxalate, and then analyzed by Beckman AU5800 automatic biochemical analyzer (The kit was provided by Beckman Coulter,

American.) to determine 120 min postprandial blood glucose (PPG) using hexokinase method.

### **Measurement of carotid plaque**

The patients were on supine position, with head toward left or right. Indicators to be determined: inner diameters of bilateral CCAs, internal CAs, external CAs, thicknesses of tunica media, maximum flow rates and resistance indexes of CCAs, resistance indexes of internal CAs, and sizes of bilateral plaques. The thickness of tunica media of carotid artery where was 15-20 mm away from junction of internal and external carotid arteries were determined. The intimal thickening was defined as intimal thickness  $>1.0$  cm but  $<1.3$  cm. The plaque was defined as intimal thickness  $>1.3$  cm.

### **Statistical analysis**

For data of patients with diabetes, the t test was used to determine the statistical differences in blood HCY, IL-8, and conditions of carotid arteries indicated on ultrasound between patients with carotid plaque and those without plaque. Also, for data of patients with carotid plaque, the t test was used to determine the statistical differences in blood HCY, IL-8, and conditions of carotid arteries indicated on ultrasound between patients with diabetes and those with hypertension. The correlations between blood HCY and IL-8 conditions of carotid arteries shown on ultrasound were analyzed.

## **Results**

### **Blood glucose and blood pressure**

For two groups with plaque, patients with hypertension had significantly higher systolic pressure and diastolic pressure than patients with diabetes ( $p<0.001$  and  $p<0.001$ , respectively). For two groups with diabetes, the fasting and postprandial blood glucose were not significantly different between patients with plaque and those without plaque ( $p=0.124$  and  $p=0.078$ , respectively). For two groups with plaque, the diabetes group had significantly higher fasting blood glucose, 2-h postprandial blood glucose and glycated hemoglobin than the hypertension group ( $p<0.001$ , respectively) (Table 1).

### **Comparison of plasma HCY and IL-8**

For two groups with diabetes, the patients with carotid plaque had significantly elevated level of plasma HCY when compared to those without carotid plaque, reaching up to  $17.58 \pm 5.71$   $\mu\text{mol}/\text{L}$  ( $p<0.001$ ). In two groups with plaque, those with diabetes had significantly elevated level of plasma HCY than those with high blood pressure ( $p<0.001$ ). For two groups with diabetes, the patients with carotid plaque also had significantly elevated level of IL-8 when compared to those without carotid plaque, reaching up to  $79.69 \pm 24.85$   $\text{pg}/\text{ml}$  ( $p<0.001$ ). In two groups with plaque, those with diabetes had

significantly elevated level of IL-8 than those with high blood pressure ( $p < 0.001$ ) (Table 2).

**Table 1.** Comparison of blood pressure and glucose metabolism among three groups.

	With diabetes and plaque n=163	With diabetes and without plaque n=123	With hypertension and plaque n=57
BMI	20.74 ± 1.84	20.81 ± 1.37	19.98 ± 1.37
Waist circumference (cm)	87.01 ± 7.48	86.59 ± 4.71	86.74 ± 7.14
Systolic pressure (mmHg)	131.61 ± 12.64	127.40 ± 10.40	151.21 ± 22.05**
Diastolic pressure (mmHg)	77.64 ± 8.76	79.05 ± 7.07	85.21 ± 13.76**
Fasting blood glucose (m mol/L)	7.58 ± 2.64	8.06 ± 2.49	5.11 ± 0.52**
2-h postprandial blood glucose (mmol/L)	12.20 ± 3.49	12.90 ± 3.10	6.97 ± 1.72**
Glycated hemoglobin (%)	8.28 ± 2.21	9.13 ± 2.36	5.81 ± 0.43**

Notes: \*\*compared with the group with diabetes and plaque,  $p < 0.01$ ; BMI: Body Mass Index

**Table 2.** Comparison of plasma HCY and IL-8 among three groups.

	With diabetes and plaque	With diabetes and without plaque	With hypertension and plaque
Number of cases	163	123	57
HCY (µmol/L)	17.58 ± 5.71	11.41 ± 4.74**	12.45 ± 3.71**
IL-8 (pg/mL)	79.69 ± 24.85	16.02 ± 8.79**	64.39 ± 28.20**

Notes: \*\*compared with the group with diabetes and plaque,  $p < 0.01$ ; HCY: homocysteine; IL-8: interleukin-8

**Table 3.** Comparison of carotid plaque among three groups.

	With diabetes and plaque	With diabetes and without plaque	With hypertension and plaque
Number of cases	163	123	57
Thickness of tunica media of RCCA (mm)	0.90 ± 0.16	0.77 ± 0.11**	0.91 ± 0.15
Resistance index of RCCA	0.76 ± 0.05	0.74 ± 0.04**	0.75 ± 0.06
Thickness of tunica media of LCCA (mm)	0.92 ± 0.16	0.83 ± 0.57	0.96 ± 0.16
Resistance index of LCCA	0.76 ± 0.05	0.73 ± 0.04**	0.75 ± 0.06

Notes: \*\*compared with the group with diabetes and plaque,  $p < 0.01$ ; RCCA: Right Common Carotid Artery; LCCA: Left Common Carotid Artery

### Comparison of carotid plaque among three groups

In two groups with diabetes, those with plaque had significantly thickened tunica media of right common carotid artery (RCCA) ( $0.90 \pm 0.16$  mm,  $p < 0.001$ ), and tendency of thickened tunica media of left common carotid artery (LCCA) ( $0.92 \pm 0.16$  mm,  $p = 0.054$ , which was not significant) when compared to those without plaque. The group with diabetes and carotid plaque had higher resistance indexes of RCCA and LCCA than the group with diabetes but without plaque, which were  $0.76 \pm 0.05$  ( $p < 0.001$ ) and  $0.76 \pm 0.05$  ( $p < 0.001$ ), respectively. For two groups with plaque, there were no significant differences in indicators between patients with diabetes and those with hypertension (Table 3).

### Analysis of correlation with HCY

The indicators that were positively correlated with HCY included IL-8 (correlation coefficient 0.474,  $p < 0.001$ ), thickness of tunica media of RCCA (correlation coefficient 0.310,  $p < 0.001$ ), resistance index of RCCA (correlation coefficient 0.242,  $p < 0.001$ ), resistance index of LCCA (correlation coefficient, 0.253  $p < 0.001$ ).

### Discussion

#### Whether does HCY aggregate VEI in diabetes

This study aimed at investigating of the relationship between HCY and VEI in diabetes and relevant mechanism for such relationship. In 1969, McCully conducted a vascular

pathological study on patients with congenital abnormal metabolism and homocysteinuria and first proposed that high blood HCY might play an important role in atherosclerosis [1]. Welch et al. [2] conducted a methionine challenge test in patients with early vascular disorders (n=123) and healthy control subjects (n=27) and found that hyperhomocysteinemia was observed in 42% of patients with cerebrovascular disorders, 28% of patients with peripheral vascular diseases and 30% of patients with coronary artery heart diseases (CHD), and that the risk for CHD was 24 times higher in patients with hyperhomocysteinemia when compared to the control group.

In recent years, hyperhomocysteinemia has attracted special attention in the study regarding the diabetes-related risk factors for cardiovascular diseases. Agullo-Ortuno et al. [3] determined the level of HCY and other biochemical indicators in 57 cases with type I diabetes, 32 cases with type II diabetes and 54 control subjects, and also investigated whether plasma HCY was correlated with major blood vessel disease, kidney disease, retinopathy and neuropathy. The results showed that diabetic individuals had significantly higher level of HCY than normal population ( $11.7 \pm 5.4$  vs.  $10.1 \pm 2.4$   $\mu\text{mol/L}$ ,  $p < 0.05$ ). Quan et al. [4] determined the plasma level of HCY in 30 normal subjects and 78 cases with type II diabetes (including 38 cases with major vascular complications) using enzyme-linked immunosorbent assay. The results indicated that high plasma HCY level was correlated with type II diabetes and its major vascular complications.

### ***Is there any correlation among HCY, diabetes and VEI?***

For two groups with diabetes that showed no difference in indicators for glucose metabolism in this study, patients with carotid plaque had significantly higher level of plasma HCY than those without plaque ( $p < 0.001$ ), revealing that HCY did cause endothelial injury in diabetes. In patients with carotid plaque, patients with diabetes had significantly higher level of plasma HCY than those with hypertension ( $p < 0.001$ ). Also, this study found that, in patients with endothelial injury, patients with diabetes had significantly higher plasma HCY than those with hypertension.

### ***What vascular injury is triggered by HCY in diabetes?***

In two groups with diabetes, the patients with plaque had significantly thickened tunica media of RCCA ( $p < 0.001$ ), and tendency of thickened tunica media of LCCA ( $p = 0.054$ ) when compared to those without plaque. The group with diabetes and carotid plaque had higher resistance indexes of RCCA and LCCA than the group with diabetes but without plaque, and had vascular injury characterized by thickened tunica media and higher resistance indexes of CCAs.

A number of reports have indicated that HCY can (1) cause morphological change of endothelial cells cultured *in vitro* [5], and significantly inhibit growth and proliferation of endothelial cells, which may be correlated with biological molecules

including HSP70, Annexin II, NO, prostaglandins (PGs), endothelin (ET), macrophage inflammatory protein I a and histamine [6-8]; (2) cause injury to vascular smooth muscle cells. HCY can promote smooth muscle cells enter from phase G1 to phase S. The microscopic examination reveals swelling of smooth muscle cells, expansion of endoplasmic reticulum, edema of mitochondria and increase in lysosome, indicating that HCY can promote proliferation of vascular smooth muscle cells [9]. Growth and proliferation of endothelial cells as well as proliferation of vascular smooth muscle cells are adequate to cause thickened tunica media and higher resistance indexes, which has been evidenced by the morphological change indicated on ultrasound of carotid artery performed in this study.

### ***What is the mechanism involving VEI triggered by HCY in diabetes?***

IL-8 is a cytokine expressed by macrophages and epithelial cells. It binds to specific receptor to exhibit chemotactic effect on neutrophils, thereby regulating inflammatory reaction [10]. IL-8 also has strong angiogenesis promoting effect. It represents the family of chemotactic factors [11]. Researchers worldwide have great interest in the mechanism by which IL-8 plays a role in the regulation of reproductive physiology and pathology. IL-8 has two types of receptors [12]: one only binds to IL-8, and the other one can bind to other chemotactic factors to attract and activate neutrophils [13]. Neutrophils have morphological changes after contact with IL-8, and directionally migrate to the target site and release a range of active products. These effects can cause local inflammation that damage cells. In two groups with diabetes in this study, the patients with carotid plaque had significantly higher level of IL-8 than those without carotid plaque. In two groups with plaque, the patients with diabetes had significantly elevated blood IL-8 than those with hypertension, demonstrating that VEI in diabetes was correlated with IL-8.

In the platelet aggregation test, the group with plaque had significantly higher platelet level, maximum aggregation ratio and slope than the group without plaque. The group with plaque had less maximum aggregation time than the group without plaque. Some experiment showed that HCY correlated with abnormal platelet function [14]. Platelet produces NO with action of NOS [15]. NO induces higher level of cGMP in platelets through activation of platelet guanylate cyclase, leading to inhibition of blood glucose adhesion and aggregation functions of platelet [16].

HCY can reduce the transportation of L-Arg in platelets. Normally, acetyl choline (ACh) stimulation can significantly increase activity of NOS in platelet. However, in the presence of HCY [17], the increased activity of NOS after ACh stimulation was significantly lower when compared with the control group. Also, the production of NO by platelets and the level of cGMP were lower when compared to the control group. These results indicate that the presence of HCY affect two factors including L-Arg transportation and activity of NOS which limit the rate of production of NO by platelets, hence

reducing the production of NO. The reduced production of NO decreases negative feedback regulation on adhesion and aggregation of platelets, so the reactivity of platelets increases, and the adhesion and aggregation of platelets also increases. The findings indicate that abnormal functions of platelets may be correlated with the HCY-triggered change of L-Arg/NO pathway in platelets.

In summary, HCY can aggregate VEI in patients with diabetes, cause thickened tunica media and trigger increased resistance index of artery. It may cause VEI in patients with diabetes through the effects of the cytokines such as IL-8.

### **Acknowledgements**

The study was supported by Shanghai Municipal Commission of Health and Family planning (2015ZB0502).

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