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The Buffering Role of HDL in Balancing the Effects of Hypercoagulable State in Type 2 Diabetes

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Abstract: Diabetes mellitus as a heterogeneous disease along with hyperglycemia causes quite many acute and chronic complications including cardiovascular complication. Cardiovascular complications are caused because of numerous factors such as increased Fasting Blood Sugar (FBS), Triglyceride (TG), hypercholesterolemia, hypercoagulable state and a change in balancing lipoproteins including Low Density Lipoproteins (LDL) and High Density Lipoproteins (HDL). Because of probable role of HDL in prevention of cardiovascular complications and its antithrombotic role in diabetics, we have studied lipoproteins and HDL in particular, effect on coagulation parameters which may potentially lead to cardiovascular complications in type 2 diabetics. In this study, 60 type 2 diabetics in early stage of diabetes were compared statistically with 50 healthy subjects in terms of biochemical factors of: FBS, TG, VLDL, LDL, coagulation parameters of: Partial thromboplastin time (PT), Activated Partial Thromboplastin Time (APTT), specific activity of coagulation factors and then the correlation between the biochemical and coagulation parameters was measured using Pearson's correlation coefficient. Our findings showed that FBS, TG, VLDL and coagulation factors of: II, IX, X, XI in diabetics had increased significantly compared with healthy subjects. They also indicated that APTT and therefore, the intrinsic coagulation pathway in diabetics prolonged in comparison with the healthy individuals. There were no other significant differences in the measured parameters between two groups. On the other hand, by studying biochemical and coagulation factors, it was shown that there was a positive significant correlation among FBS, cholesterol and HDL with the coagulation factors of II, V, IX, X, XI. There was, interestingly, a negative significant correlation between HDL and APTT. The observed correlation between coagulation factors and HDL, regardless of the no significant difference of HDL in the two groups, shows that probably the hypercoagulable state as a result of hyperglycemia led to plasma increase of HDL. In other words, HDL, probably, because of hypercoagulable state, intended to remove their destructive effects of hypercoagulable state and then correlated with them.

Key words: High density lipoprotein, coagulation factors, cardiovascular complications, diabetes mellitus

INTRODUCTION

Diabetes Mellitus (DM), as a current complicated disease, is considered as one of the main causes of cardiovascular complications (Laing *et al*., 2003; Soedamah-Muthu *et al*., 2006; Titty *et al*., 2008; Kiencke *et al*., 2010; Sayyad *et al*., 2011). It has been proved that irregular plasma glucose, hyperglycemia in particular, results in many acute and chronic diseases like nephropathy, retinopathy, neuropathy, atherosclerosis and blood pressure (Deckert *et al*., 1996; Joshi *et al*., 1999; Bertoni *et al*., 2001; Hill *et al*., 2001; Behnam-Rassouli *et al*., 2010). In terms of pathophysiology, studies show that the two main types of DM (DM1 and DM2) cause a change in balancing of metabolites such as carbohydrates, lipids and blood coagulation factors (Ghattas *et al*., 2008; Mard-Soltani *et al*., 2011a, b, c; Dayer *et al*., 2011) and subsequently bring about complications like microvascular and cardiovascular complications

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(Vijan *et al*., 1997; Behnam-Rassouli *et al*., 2010). It has also been shown that cardiovascular disease are not mono factorial complication, are not related to hyperglycemia conditions only and that other various factors including change in coagulation homeostasis and lipoproteins balancing are also involved (Ernst and Resch, 1993; Heinrich *et al*., 1994; Assal *et al*., 2007; Tellis *et al*., 2009). Today, change in coagulation homeostasis and hypercoagulable state in the two types of DM patients, especially T2DM; have been shown to be correlated with cardiovascular complications (Alzahrani and Ajjan, 2010; Tas *et al*., 2011). Prescription of anticoagulant medicines like aspirin to prevent intravascular thrombosis in cardiovascular patients proves the role of coagulation factor in cardiovascular abnormality (Gharebaghian and Eghtesadi-Araghi, 2006; De Berardis *et al*., 2010). Regarding the multi factorial cause of cardiovascular complications in diabetics, studies show that there are preventive biomarkers which reveal the risk of cardiovascular complications (Sharrett *et al*., 2001; Kiencke *et al*., 2010). Two such markers are High Density Lipoprotein Cholesterol (HDL-C) and coagulation factors (Assmann *et al*., 1996; Asztalos and Schaefer, 2003). Many studies about HDL shows that this lipoprotein is well known as a heterogenic class of plasma lipoproteins with high viscosity and small size which , besides carrying of blood cholesterol from other organs to liver and to other reproductive organs such as testes and ovaries, also participates in carrying many plasma proteins (Asztalos and Schaefer, 2003; Karlsson *et al*., 2005; Kontush and Chapman, 2006a; Vaisar *et al*., 2007; Davidson *et al*., 2009).

The studies confirm that normal level of HDL reduces risk factors in cardiovascular complications (Chapman *et al*., 2004; Barter and Kastelein, 2006; McTaggart and Jones, 2008). In addition, epidemiologic studies have shown that HDL reduction is a basic, strong and an independent factor in predicting complications of coronary arteries (Luc *et al*., 2002) and in general, an increase in HDL lipoprotein containing apo-AI, apo-AII reduces coronary diseases (Birjmohun *et al*., 2007; Wan der Steeg *et al*., 2008). On the other hand, the studies show that treatments along with HDL increase result in the reduction of cardiovascular complications and subsequent deaths (Goldenberg *et al*., 2009). Recent studies confirm the fact that HDL has an intravascular metabolism and anti-coagulation characteristic and includes two subtypes (Chapman *et al*., 2010), The large subtype is light and is full of cholesterol esters (HDL-2) and the small subtype with little cholesterol ester and much protein (HDL-3) (Kontush and Chapman, 2006a). In previous studies, these subtypes are analyzed into their constituents by different methods including two-dimensional electrophoresis and immuno-affinity chromatography and etc, the constituents of these lipoproteins gradually emerge (Kontush and Chapman, 2006a). It has now been proved that besides its anti-arthritis characteristic, HDL plays a role as a co-factor for protein C (which is a regulating protein in coagulation and fibrinolytic pathways) anti-coagulation activity (Osei *et al*., 1991; Lewis *et al*., 1993; De Vegt *et al*., 1998; Assmann and Nofer, 2003). According, to the studies carried out on the protective role of HDL in developing cardiovascular complications, however, there are contradicting reports regarding the effect of lipoproteins on the development of cardiovascular complications in diabetics and some studies reject HDL contribution to the prevention of cardiovascular complications (Lewis *et al*., 1995). Regarding the probable roles of HDL in prevention of cardiovascular complications in diabetics and the results obtained by our research team in relation to the increase of coagulating factors in T2DM patients (Mard-Soltani *et al*., 2011a, b, c), we in this study investigate the probable role of plasma lipoproteins specially HDL in changing coagulation parameters which may potentially cause cardiovascular complication in T2DM. That is why we have measured biochemical factors: FBS, fasting triglyceride (FTG), plasma lipoproteins: include Low Density Lipoprotein (LDL), Very Low Density Lipoprotein (VLDL) and HDL and the specific activity of coagulation factors for the control and the diabetic subjects. Then, we compared the two groups statistically in terms of the above mentioned parameters and measured the correlation between biochemical parameters and the coagulation parameters in T2DM.

MATERIALS AND METHODS

Subjects selection: In this study, 60 T2DM patients were selected from among 2300 patients in The Great Hospital of Dezful (in Khuzestan Province, Iran) and were compared with 50 healthy hospital employees of the same hospital who had no history of acute and chronic diseases in terms of biochemical and hematologic parameters. T2DM patients were selected according to their history in Diabetes Clinic of Dezful. All these patients were suffering from the DM for less than three years. They were all diagnosed as T2DM patients in the years of 2007-2011. They had the age range of 45-60. All these participants were already informed of the procedure the study would have been implemented. If the patients had any history of cardiovascular complications, vascular disease, homeostasis disorder, nephropathy, retinopathy, insulin therapy, psychological-mental disorder or

smoking, they would have been excluded from these assessments. The only medication used by the patients was a single anti-diabetic Metformin pill taken daily. The experimental procedures were followed according to the guidelines approved by the Research Ethical Committee of the Faculty of Medical Sciences of Dezful.

Sampling preparation: Blood samples were drawn in the morning of the test day when the subjects had been fasting for 8 to 12 h. In the first phase of the study, 9 mL of blood was drawn from each subject and tested for biochemical parameters in the standard medical diagnostic laboratory of Dezful Hospital. To measure coagulation parameters, 2 mL from the same 9 mm was put in a plastic tube containing 0.2 cc of sodium citrate with 0.106 mol and was frozen in less than 2 h in dry-ice condition and conveyed to hematology laboratory of hospital of Shafa in Ahvaz. Then, plasma samples were prepared by centrifuging twice in 1500 xg in the temperature between 15° to 18° centigrade and were stored in −70°C. Next, measuring coagulation factors were conducted at proper times. All methods were assayed in a duplicated method. Any plasma sample was discarded if it had hemolysis and sampling was repeated for that patient on the next day.

Analytical methods: Weight and height of the subjects were measured in light clothes without shoes. Their body mass index (BMI) was calculated by their weight (kg) divided on the square meter (m^2) of height. The Fasting Blood Glucose (FBS) of patients was measured by enzymatic method of GOD/PAP using the laboratory kit made by Pars Azmun Company (Pars Azmun, Karaj, Alborz Province, Iran). Duplicated glucose level of =6.1 mmol L^{-1} or 110 (mg dL^{-1}) was considered as an indicator of being diabetic. The blood FTG, LDL (LDL-C), HDL (HDL-C) and total plasma cholesterol level (TC) were assessed using calorimetric kits made by Pars Azmun Co. Activated Partial Thromboplastin Time (APTT) and Prothrombin Time (PT) were measures by ACL 8000 coagulation analyzer (Beckman Coulter, Fullerton, California) using APTT-SP kits and PT-Fibrinogen HS PLUS made by HemosIL Company, respectively. Respectively, Von Willebrand factor specific activity and fibrinogen (F-I) concentration in blood plasma were measured by HemosIL von Willebrand factor antigen and Fibrinogen HS PLUS kits made by HemosIL Company, using ACL 8000 Analyzer. Moreover, the specific activities of coagulation factors of: II, VII, VIII, IX, X and XI were measured in citrated plasma using standard HemosIL Deficiency kits of: II, V, VII, VIII, IX, X and XI and by the use of ACL 8000, respectively. All experimental procedures involving human participants were with due attention to the guidelines approved by the Research Ethical Committee of the Faculty of Medical Sciences of Dezful.

Statistical analysis: The data were analyzed statistically. Since the number of subjects was more than thirty, the distribution was considered as normal. The difference was investigated in the experimental and the control group in terms of demographic, biochemical and coagulation parameters using independent t-test and was presented as Means±SEM. Next, Pearson's correlation coefficient was calculated among all the obtained measures of plasma lipoproteins and the specific activity of coagulation factors in diabetics. Statistical analysis was performed using the Statistical Package for the Social Science (SPSS-PC, version 15. SPSS, Inc., Chicago, IL). The proportion of the missing data was at most 7.5% in all parameters which did not affect the results. The probability level for all statistical results was $p<0.05$.

RESULTS

In Table 1, there was a comparison between the T2DM group and the control group using independent t-test in terms of biochemical and demographic parameters. FBS of T2DM patients showed an increase compared with the control group with $p<0.001$. Moreover, in terms of TC, LDL and HDL there was no significant difference between the two groups but there was a significant difference in VLDL amount ($p<0.01$).

Table 2 presents the data of the T2DM patients and the healthy subjects in terms of coagulation parameters. In this study, coagulation factors of II, VII, IX, X and XI in T2DM patients showed significant increase in comparison with healthy subjects. In addition, the specific activity of coagulation factors VIII in the T2DM group showed significant decrease in comparison with control group. In terms of coagulation V factor there was no significant difference between the two groups. Also, APTT prolonged significantly in the control group compared with the diabetic group; however, in terms of PT and PT_{INR}, there was no significant difference between two groups.

In Table 3, Pearson correlation coefficient data between biochemical factors (FBS, TG, TC, LDL, HDL-C and VLDL) and coagulation parameters in type 2 diabetics are presented. And the findings of the presented data show that FBS have a significant positive correlates with specific activity of coagulation factors of II, V, VIII, IX, X and XI. Also, FBS negatively correlates with time period of APTT. As shown in Table 3, there is a positive

Table 1: Comparison of demographic and biochemical parameters in diabetics and Control groups

	Diabetics (n = 60)	Control (n = 50)	Significance
Gender (Male%)	48.33	50	ns
Age (Years)	55.73±2.330	53.43±2.110	ns
BMI (Kg m^{-2})	29.02± 1.32	25.17± .490	p<0.01
Chol (mg dL^{-1})	198.5±7.6100	188.36±8.920	ns
TG (mg dL^{-1})	235.04±13.79	149.00±21.70	p<0.05
FBS (mg dL^{-1})	162.58±14.02	97.92±13.23	p<0.01
HDL-C (mg dL^{-1})	62.86±2.420	57.20±5.180	ns
LDL (mg dL^{-1})	93.63±1.800	92.25±3.120	ns
VLDL (mg dL^{-1})	45.19±2.050	31.66±3.420	p<0.01

ns: Non significant

Table 2: Comparison of coagulation parameters in diabetics and Control groups

Parameter	Diabetics (n = 60)	Control (n = 50)	Significance
vWF (IU dL^{-1})	107/03±3.44	96.58±7.35	ns
F-I (g L^{-1})	3.42±.32000	3.80±.2300	ns
F-II (IU dL^{-1})	103.12±1.43	95.08±1.83	p<0.01
F-V (IU dL^{-1})	88.27±1.800	90.73±.820	ns
F-VII (IU dL^{-1})	96.46±1.790	93.21±1.32	p<0.05
F-VIII (IU dL^{-1})	61.05±5.710	91.90±6.30	p<0.001
F-IX (IU dL^{-1})	112.38±3.52	90.11±6.34	p<0.01
F-X (IU dL^{-1})	105±2.46000	97.72±3.27	p<0.001
F-XI (IU dL^{-1})	104.53±3.22	79.08±6.67	p<0.001
APTT	38.94±.6600	29.07±.790	p<0.001
PT(sec)	12.56±.1900	12.51±.170	ns
PT_{INR}	0.97±.02500	0.94±.0120	ns

Table 3: Pearson correlation coefficient between biochemical and coagulation parameters in diabetics (n = 60)

		FBS	Chol	TG	HDL-C	LDL-C	VLDL
vWF	Correlation	0.148	-0.013	0.082	-0.127	-0.148	0.144
	Sig. (2-tailed)	0.396	0.938	0.639	0.460	0.397	0.402
F-1	Correlation	0.300	0.224	0.231	-0.196	0.168	0.249
	Sig. (2-tailed)	0.080	0.189	0.181	0.252	0.327	0.143
F-II	Correlation	0.389*	0.340*	-0.118	0.449**	0.183	-0.030
	Sig. (2-tailed)	0.016	0.034	0.481	0.004	0.270	0.857
F-V	Correlation	0.488**	0.324*	-0.014	0.486**	0.121	0.051
	Sig. (2-tailed)	0.002	0.047	0.934	0.002	0.477	0.762
F-VII	Correlation	0.300	0.123	0.342*	-0.013	-0.230	0.391*
	Sig. (2-tailed)	0.071	0.463	0.038	0.938	0.171	0.015
F-VIII	Correlation	0.387*	0.153	0.170	0.134	0.028	0.197
	Sig. (2-tailed)	0.029	0.396	0.353	0.457	0.881	0.271
F-IX	Correlation	0.407*	0.578**	0.184	0.484**	0.414*	0.235
	Sig. (2-tailed)	0.015	0.000	0.290	0.003	0.014	0.168
F-X	Correlation	0.536**	0.397*	0.018	0.402*	0.307	0.017
	Sig. (2-tailed)	0.001	0.012	0.917	0.011	0.061	0.916
F-XI	Correlation	0.546**	0.446**	0.016	0.513**	0.318	0.015
	Sig. (2-tailed)	0.001	0.007	0.928	0.002	0.067	0.931
APTT	Correlation	-0.334*	-0.270	0.067	-0.434**	-0.270	0.043
	Sig. (2-tailed)	0.041	0.097	0.690	0.006	0.101	0.797
PTs	Correlation	-0.266	-0.284	-0.183	0.125	0.066	0.260
	Sig. (2-tailed)	0.107	0.0800	0.271	0.447	0.695	0.109
PTinr	Correlation	-0.295	-0.187	-0.192	0.060	0.084	0.279
	Sig. (2-tailed)	0.073	0.253	0.249	0.718	0.615	0.085

*, **Significant at p<0.05 and <0.01

significant correlation between HDL-C and the specific activity of coagulation factors of: II, V, IX, X and XI. The results showed that HDL-C and APTT have significant negative correlation. Also, the LDL and specific activity of coagulation factors of XI and X show positive significant correlation. Moreover, there was a positive significant correlation between TC and fibrinogen and the specific activity of coagulation factors of II, V, IX, X and XI. Also, there was a positive significant correlation between the fibrinogen and the specific activity of coagulation factor of VII.

DISCUSSION

Regarding the findings of our study, the elevation of FBS and VLDL in T2DM were observed. In general, T2DM patients had more fasting and random blood glucose, compared with healthy subjects (Mard-Soltani *et al*., 2011a, b, c). This difference was mostly due to insulin reduction, delay of insulin excretion, or defect in insulin receptors (GLUT-2) (Osei *et al*., 1991; De Vegt *et al*., 1998; Akbarzadeh *et al*., 2007). Numerous studies have shown that reduce excretion and/or dysfunction of insulin, besides increasing blood glucose, causes TG increase in T2DM patients (Osei *et al*., 1991; De Vegt *et al*., 1998; Akbarzadeh *et al*., 2007). On the other hand, clinical evaluations strongly confirm that the amount of lipoproteins rich in TG like chylomicron and VLDL increases after nutrition and quickly returns to its normal level again (Lewis *et al*., 1993, 1995; Malmstrom *et al*., 1997, 1998; Adiels *et al*., 2007). These lipoproteins are hydrolyzed mainly by lipoprotein lipase existing in the capillaries of fat tissues (Kiens *et al*., 1989; Merkel *et al*., 2002). The activity of lipoprotein lipase causes conversion of VLDL and chylomicron to TG and fatty acids (Ginsberg, 1991; Merkel *et al*., 2002). Physiological studies confirm that the function of this enzyme is closely dependant on insulin (Pollare *et al*., 1991). Therefore, a reduction in insulin in T2DM reduces the activity of lipoprotein lipase as well and increases VLDL and chylomicron (Badin *et al*., 2011). The results in Table 1 and the parameters for the selection of subjects of this study show that the lipopatients have developed T2DM. Moreover, lack of any statistically a significant difference in TC level between the two study groups and because the T2DM patients were not already suffering from any acute and chronic disorders, confirms the fact that they are in early stages of T2DM.

In terms of coagulation parameters of the diabetics and healthy subjects and the studies done by our team in the past confirms the evaluation of coagulation factors in T2DM patients (Mard-Soltani *et al*., 2011a, b, c). The results showed that, the coagulation factors, except I, V, vWF, in T2DM patients increased significantly and only the coagulation co-factor VIII decreased significantly. Moreover, our results indicated that the time period (APTT) in the patients group had prolonged significantly and the reason was probably the reduction of suppressive role of c-factors V, VIII and the increase of inefficient intravascular thrombosis or strengthening of fibrinolytic

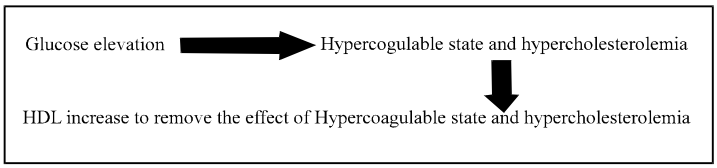

Fig. 1: The relationship between glucose, hypercoagulable state and the effect of these factors on HDL

pathway (Mard-Soltani *et al*., 2011a, c). Prolonging of time period (APTT) and involvement of intrinsic coagulation pathway has also been confirmed in our pervious studies (Mard-Soltani *et al*., 2011a, c). The findings also indicate that the FBS and coagulation factors had the highest positive significant correlation and plasma glucose level is probably the most important parameter in hypercoagulable state in T2DM patients (Mard-Soltani *et al*., 2011c). Also, High correlation between glucose and specific activity of coagulation factors has already been shown in our previous study (Mard-Soltani *et al*., 2011b, c).

On the other hand, our studies show that there are meaningful correlations between coagulation factors and the level of TC and HDL-C and this is exciting since before to sense the significant difference of HDL-C in diabetics in comparison with healthy subjects, the coagulation factors in diabetics had shown significant increase compared with healthy subjects, an issue not happening for glucose. Significant positive correlation between HDL-C and coagulation factors shows the possible effect of coagulation factors on HDL-C. That is, despite the significant difference between coagulation factors of healthy subjects and diabetics in the early stage of the disease, there is yet no significant difference between the amount of HDL-C and cholesterol in the two groups. The precedence of changes in coagulation factors over the observed changes in HDL-C probably indicates the buffering role of HDL in balancing the hypercoagulable state and then a reduction in cardiovascular risks Fig. 1.

Our findings also show that HDL effect on reducing cardiovascular complications in diabetics can be partly because of HDL effect on specific activity of coagulation factors. The observed correlation in our studies confirm that HDL tries to balance the coagulation factors level involved in coagulation cascade specially the intrinsic coagulation cascade, however, with its microscopic increase, it could not have controlled the macroscopic change of coagulation factors. Probably, secondary complications in cholesterol metabolism and the correlation between cholesterol and coagulation factors are correlated with the buffering role of HDL to remove the destructive effect of hypercoagulable state. This point is confirmed by proteomic analysis of HDL which show that 75 distinctive proteins, of which many are unknown, exist in HDL and are probably carried by it (Vaisar *et al*., 2007; Davidson *et al*., 2009).

Also, numerous studies show that all human HDL subsets which contain apo-AI have the susceptibility of absorbing cholesterol and are anti-oxidants, anti-inflammation, epitasis and anti-coagulation. They possibly have this role because of the effect of proteins in them (Kontush and Chapman, 2006a, b; Briel *et al*., 2009; Oslakovic *et al*., 2010).

Moreover, some studies show that 20% increases in blood HDL concomitant with a reduction in macrophages size resulted in fewer adhesion to adhesive molecules of VCAM-1 on the endothelium, involved in formation of intravascular plaques (Nicholls *et al*., 2007). In many clinical studies, it has been confirmed that HDL increase is concomitant with a reducing development of atherosclerosis of coronary arteries and cardiovascular complications (Chapman, 2006; Nissen *et al*., 2006; Brown and Zhao, 2008). Based on the findings of this study and the previous ones, we can conclude that HDL, because of its high correlation with most coagulation factors, probably functions as a buffer to absorb coagulation factors. The findings of this study gives us the promising perspective of manipulating HDL and its proteins to reduce risks of developing cardiovascular and homeostasis complications in patients, specially T2DM ones. Of course, to study the exact mechanism of the relationship between HDL and coagulation parameters or the unknown proteins, we need more researches using different special techniques to study proteins, what which is currently underway by our team research.

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