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High density lipoprotein - 3 in diabetic retinopathy patients: relationship to total antioxidant capacity and nitric oxide level

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糖尿病视网膜病变患者高密度脂蛋白-3 和总抗氧化能力以及 NOx 水平分析

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摘要
目的:比较糖尿病视网膜病变组与对照组数据,以研究高密度脂蛋白-3(HDL_3)、NOx 和总抗氧化状态之间的关系。
方法:前瞻性病例对照研究。106 例受试者分为 3 组,84 例 2 型糖尿病有或无视网膜病变患者,对照组为 22 例正常人。患者均行血清高密度脂蛋白-3 的浓度检测和血清 NOx 水平测定。用铁还原法(FRAP) 测量血浆总抗氧化能力。
结果:糖尿病患者(DM)空腹血糖、糖化血红蛋白、甘油三酯显著高于对照组。正常人中 HDL_3 水平为 14.4(12.0) mg/dL,糖尿病视网膜病变患者为 18.1(12.6) mg/dL,糖尿病无视网膜病变患者为 14.0 (12.5) mg/dL,组间差异无统计学意义($P=0.262$)。糖尿病患者 FRAP 水平低于对照组($P=0.003$),但糖尿病视网膜病变组与非糖尿病视网膜病变组之间其差异无统计学意义。
结论:研究发现:2 型糖尿病视网膜病变患者、糖尿病无视网膜病变患者以及对照组间 HDL 和 HDL_3 水平无明显不同。HDL_3 可能无法预测糖尿病视网膜病变患者的患病风险。糖尿病患者中血清 NOx 水平较高,FRAP 水平较低。
关键词:高密度脂蛋白;糖尿病视网膜病变;一氧化氮;总抗氧化

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Abstract

• AIM: To examine the association between high density lipoprotein (HDL) 3 cholesterol, nitrite plus nitrate (NOx) and total antioxidant status in diabetic retinopathy patients compared to controls.

• METHODS: This was a prospective, case-control study. One hundred and six participants were subdivided into three groups. Eighty-four type 2 diabetes patients with and without retinopathy and 22 healthy controls were included in this study. Serum HDL_3 concentrations were assayed and serum NOx levels were determined in all patients. Total antioxidant capacity was measured using the ferric reducing power of plasma (FRAP) assay.

• RESULTS: Among the subjects with diabetes mellitus (DM), fasting glucose, HbA1c and triglycerides were significantly higher than the healthy controls. HDL_3 level was 14.4 (12.0) mg/dl in healthy subjects, 18.1 (12.6) mg/dl in the diabetic retinopathy group and 14.0 (12.5) mg/dl in diabetic patients without retinopathy, and was statistically similar between the groups ($P=0.262$). HDL level was similar between groups in our population. FRAP level was lower in patients with DM compared to healthy controls ($P=0.003$), but was not different between the DR and the non-DR groups ($P=0.913$).

• CONCLUSION: In our study, we demonstrated that HDL and HDL_3 subgroup levels didn't significantly differ between DM2 patients with DR and without DR and healthy controls. Determination of HDL_3 cholesterol, in addition to total HDL cholesterol, may not predict the actual risk for diabetic retinopathy. Serum NOx was observed to be higher in diabetic participants and FRAP level was low.

• KEYWORDS: high density lipoprotein; diabetic retinopathy; nitric oxide; total antioxidant
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INTRODUCTION

Diabetic retinopathy (DR) is one of the microvascular complications of diabetes, which is the most common cause of blindness in adults[1-2]. Many factors are reported to be associated with DR, including the duration of diabetes mellitus (DM), hypertension, obesity, sedentary life style and smoking, but it is primarily caused by the metabolic effects of chronic hyperglycemia[1-2]. Moreover, the broad spectrum of DR severity in diabetic patients with similar biochemical profiles suggests several additional environmental, genetic and epigenetic factors that influence pathogenesis of DR[3]. Therefore, it is plausible that hyperglycemic environment disturbs metabolic homeostasis and also alters various genes, including genes associated with oxidative stress[4].

Significant changes in lipid metabolism and structure have been reported as an additional risk factor for DR[5-7]. Serum lipids may contribute to microvascular dysfunction that subsequently results in the breakdown of the blood-retinal barrier and development of retinovascular lesions that occur in DR[8].

HDL particles in human plasma are highly heterogeneous in structure with different protein and lipid composition and pathophysiological significance[9]. In addition to cholesterol efflux, HDL has been shown to display a broad spectrum of metabolic activities and exhibit strong anti-oxidant effects[10]. HDL consists of two major subfractions, large, less dense HDL_2 and small, dense HDL_3[11]. However, it is not known whether the distribution of HDL subgroups is different in subjects with DR compaired to patients without retinopathy.

HDL subpopulations show differences in their anti-oxidant activity. Increased oxidative stress from excessive reactive oxygen species production is also considered to be an important contributor to DR[12]. It has been shown that HDL can stimulate nitric oxide (NO) production, which might contribute to oxidative stress. Meanwhile, increased NO levels have been shown to be associated with increased severity of diabetic retinopathy[13-14].

To prevent cells and cellular biomolecules from being damaged by reactive oxygen, plasma contains many compounds that work together. Measurement of total anti-oxidant capacity (TAC) in serum is an integrated parameter that may give information about patients' antioxidant status[15].

The objective of the present study was to examine the association between HDL_3 cholesterol, nitrite plus nitrate (NOx) and total antioxidant status [according to the ferric reducing power of plasma method (FRAP)] in diabetic retinopathy patients compared to controls.

SUBJECTS AND METHODS

Ethics clearance for the study was granted by the Regional Ethics Committee. This was a prospective, case-control study conducted at the department of biochemistry in collaboration with the ophthalmology department. DM2 patients 45-75y that attended the ophthalmology referral clinic and consented to take part in the study between January and August 2016 were recruited. We followed the tenets of the Declaration of Helsinki. Informed consent was obtained from all the participants.

All the subjects were diagnosed to have type 2 diabetes mellitus according to the 2016 American Diabetes Association standards[16]. Type 1 diabetic patients, organ failure, liver disease, stroke, recent systemic infections, and patients taking multivitamins, fibrates, statins and any amount of alcohol were excluded. DM2 patients with and without retinopathy were included in the study. DR was evaluated by experienced ophthalmologists using an indirect ophtalmoscope while the patients' pupils were dilated. DR was defined as the presence of neovascularization of the retinal vessels and the complications of this neovascularization, such as preretinal hemorrhage, vitreous hemorrhage or traction retinal detachment [17].

A total of 106 participants were subdivided into three groups. Eight-four type 2 diabetes patients with or without retinopathy and 22 healthy controls were included in this study. All our subjects had similar sedentary lifestyles.

Body mass index (BMI) was calculated as the ratio of body weight in kg/height in square meters. Fasting overnight venous blood and first morning urine samples were collected from all individuals. Serum samples were separated from the specimen within 1h after collection and stored at -80℃ if not analyzed on the day they were collected.

Serum HDL_3 concentrations were assayed using a commercial ELISA kit (Sun Red Shanghai, China) according to the manufacturer's instructions. The range of the standard curve was 10-3000 nmol/mL.

Serum nitrite plus nitrate (NOx) levels were determined in all patients who were recruited to the study. NOx levels were measured in blood obtained in the early morning. Total NOx levels were used as a surrogate marker for serum nitric oxide levels[18]. During analysis, the serum samples were first thawed, then deproteinized by adding zinc sulfate[19]. The supernatant was pipetted (100 μL) in duplicate into a 96-well ELISA plate, 100 μL of vanadium (III) chloride (8 mg/mL) was added to each well (for reduction of nitrate to nitrite) followed by the addition of 100 μL of Griess reagent (equal mixture of 1% sulphanilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl) ethylenediamine hydrochloride in distilled water). After incubation for 30min, the optical

Table 1 Characteristics of the study population

Characteristics	Healthy controls	DM retinopaty	DM without retinopathy	*P*
Number of patients (*n*)	42	42	22	
Age (a)	60.1±6.8	60.6±7.1	58.8±7.4	0.431
M/F	17/25	16/26	10/12	–
Duration of diabetes	–	15±5	12±5	
BMI(kg/m^2)	27.9±4.0	29.3±5.0	29.8±4.9	0.148
Hba1c (%)	5.6(0.77)	8.7(2.3)	8.2(2.1)	0.001[a]
Fasting glucose (mg/dL)	98(16)	192(165)	159(80)	<0.001[a]
BUN (mg/dL)	15.0±6.4	16.0±7.4	15.0±7.6	0.654
Creatinine (mg/dL)	0.9±0.2	1.0±0.4	0.8±0.1	0.027[a]
Microalbumin/creatinine(μg/mg)	11(16)	25(89)	18(54)	0.050
AST	23(8)	19(4)	20(10)	0.083
ALT	20(9)	19(8)	20(15)	0.172
T Chol (mg/dL)	207±44	206±42	227±57	0.270
Trig (mg/dL)	156(66)	174(89)	167(125)	0.027[a]
HDL(mg/dL)	52±22	53±20	47±11	0.442
Uric acid	5.3(2.2)	5.9(2.2)	4.9(2.1)	0.145
T protein	7.0(0.4)	7.0(0.5)	7.0(0.6)	0.599
CRP	3.3(3.3)	3.3(4.9)	3.9(2.4)	0.609
RDW	13.5(0.9)	13.9(1.4)	13.9(1.9)	0.111
AIP	0.14±0. 39	0.20±0.24	0.14±0.37	0.633
NOx (μmol/L)	35± 2	40±3	38±3	0.335
FRAP	1002±281	797±387	746±411	0.003[a]
HDL_3(mg/dL)	14.4(12.0)	18.1(12.6)	14.0(12.5)	0.262

Data are expressed as mean ± standard deviation when normally distributed, otherwise as median (interquartile range); BMI: Body mass index; HbA1c: Hemoglobin A1c; T Chol: Total cholesterol; Trig: Triglyceride; AIP: Atherogenic index of plasma; NOx: Nitrite plus nitrate; FRAP: Ferric reducing power of plasma; CRP: C-creactive protein; RDW: Red cell distribution width; AST: Asprtat aminotransferase; ALT: Alanin aminotransferase; BUN: Blood urea nitrogen; DM: Diabetes mellitus; [a]$P<0.05$ was considered significant for statistical analyses.

density was measured at 540nm using a Readwell Touch Elisa plate analyzer (Robonik PVT Ltd. Mumbai, india). A standard curve was plotted using $NaNO_2$ as standard.

Total antioxidant capacity was measured using the FRAP assay by the method of Benzie and Strain (1996) with minor modifications[20]. The method is based on the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) to the ferrous form (Fe^{2+}-TPTZ).

Statistical Analyses The IBM Statistical Package for Social Science software (SPSS for Windows, version 21.0, USA) was used to perform all statistical analyses. Data are expressed as mean±standard deviation (SD) when normally distributed, or median (IQR). A one-way analysis of variance (ANOVA) test was performed for significant differences among groups. The Tukey HSD was used as a post hoc test for variables with normal distribution. Nonparametric Kruskal-Wallis test was conducted for variables not following a normal distribution. Mann-Whitney test with Bonferroni correction was used for post hoc pairwise analysis of the Kruskal-Wallis test. Spearman rank correlation was used to analyze relationships between continuous variables. A $P<0.05$ was considered to be statistically significant.

RESULTS

The general profile of the enrolled participants is presented in Table 1.

With regard to age and BMI, no significant difference was observed ($P=0.431$ and $P=0.148$). Among the DM subjects, fasting glucose, HbA1c and triglycerides were significantly higher than the healthy controls. However, no significant differences were detected between the DR and the non-DR groups for fasting glucose, HbA1c or triglycerides ($P=0.226$, $P=0.154$, and $P=0.966$, respectively).

Microalbumin/creatinine was not significantly different between groups. HDL_3 level was 14.4 (12.0) mg/dl in healthy subjects, 18.1 (12.6) mg/dl in the diabetic retinopathy group and 14.0 (12.5) mg/dl in diabetic patients without retinopathy, and was statistically similar between the groups ($P=0.262$) (Figure 1).

HDL level was similar between groups in our population. FRAP level was lower in DM patients compared to healthy controls ($P=0.003$), but was not different between the DR and the non-DR groups ($P=0.913$).

No significant correlation was found between HDL_3 and continuous patient variables by Spearman's rank correlation analysis (Table 2).

DISCUSSION

In our study, we demonstrated that HDL and HDL_3 subgroup levels did not significantly differ between DM2 patients with DR and without DR and healthy controls.

Clinical studies have come to discordant conclusions on the

Table 2　Correlation between different variables and HDL_3 using Spearman rho correlation coefficient

Paraments		BMI	Microalb/Cr	HDL_3	CRP	FRAP	NOx	Duration of diabetes mellitus
HbA1c	*r*	0.224	0.273	0.057	0.141	-0.155	0.300	-0.108
	P	0.018[a]	0.006[a]	0.580	0.046[a]	0.126	0.040[a]	0.407
BMI	*r*		0.093	0.110	0.264	0.036	0.047	-0.155
	P		0.356	0.265	0.004[a]	0.699	0.643	0.196
Microalb/Cr	*r*			0.137	0.256	0.00[a]	0.058	0.058
	P			0.201	0.011[a]	0.980	0.598	0.681
HDL_3	*r*				-0.050	0.135	-0.035	-0.152
	P				0.618	0.193	0.743	0.260
CRP	*r*						0.150	-0.026
	P						0.148	0.844

BMI: Body mass index; Microalb/Cr: Microalbumin/creatinine; HDL_3: High density lipoprotein-3; NOx: Nitrite plus nitrate; FRAP: Ferric reducing power; CRP: C-reactive protein; [a]$P<0.05$ was considered significant for statistical analyses.

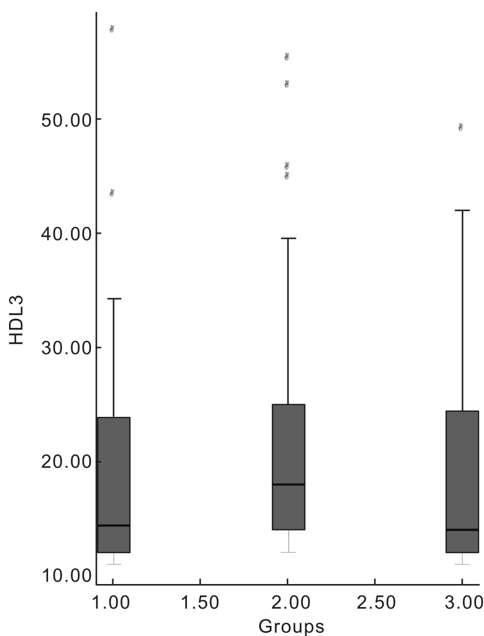

Figure 1　HDL_3 levels in different groups HDL_3 levels in patients with diabetic retinopathy (Group 2) and diabetic patients without retinopathy (Group 3) are compared with controls (Group 1). Box plot showing median (line), interquartile range (boxes), and 5% to 95% percentile (whiskers), and outliers (dots).

prognostic value of HDL subclasses in DM2 patients. It has been demonstrated that the HDL_3 level was significantly increased in prediabetic subjects compared with controls[21] and among DM2 patients[22]. However, in Japanese patients higher HDL_3 cholesterol was not associated with DM2 risk[11]. There is evidence that the HDL_2 fraction has a protective role for coronary heart disease in DM2 patients[23-24].

Meanwhile, similar to our results, HDL_3 cholesterol levels were shown to be unaltered in comparison to nondiabetic controls among coronary artery disease patients[23]. Serum HDL_3 subgroups with type 2 diabetic microvascular complications have not been well studied, especially in DR.

Although all ethnic groups are susceptible to the established risk factors of DR, such as duration of the disease, severity of hyperglycemia and hypertension, ethnicity specific risk factors exist[24-25]. Serum lipid levels may also affect such different populations at different levels[24-25]. Intra-retinal extravasation and modification of low density lipoprotein (LDL) have been implicated in diabetic retinopathy. It has been shown that modified LDL is associated with increased oxidative stress in the retina, which results in pericyte loss, retinal injury and progression of DR[26].

Hyperglycemia induced oxidative and nitrosative stress can lead to capillary cell dysfunction and retinal damage[27-29]. The retina is more susceptible to oxidative and nitrosative stress since it has the highest oxygen uptake and glucose oxidation than any other tissue. Therewithal, it has been observed that hyperglycemia induces expression of nitric oxide synthase (eNOS) and increases formation of superoxide anion and nitrotyrosine (O_2^-). Reaction between superoxide and NO forms potent oxidant radical peroxynitrite ($ONOO^-$) which also lead to apoptosis of human retinal endothelial cells and retinal microvascular degeneration[28-29].

The earliest and most significant change in diabetic retinopathy (DR) is blood-retinal barrier (BRB) dysfunction[30]. Following blood retinal barrier damage, extravasation of LDL in the extracellular tissue modifies LDL further through oxidation and glycation, making it more toxic towards retinal cells. Studies have shown a significant relationship between ox-LDL levels and the presence of DR[31].

Recent studies reveal that HDL_3 potentially protects human microvascular endothelial cells against primery apoptosis induced by oxidized LDL and intracellular ROS generation[32].

Specific molecular lipid and protein species are distributed non-uniformly across HDL particle subpopulations[33-34]. Several phospholipids and sphingolipids can directly affect antioxidative and anti-inflammatory effects of HDL, acting in part via modulation of physical properties[35-36]. Sphingosine-1-phosphate is enriched in HDL_3 that play a role in the cytoprotection from apoptosis provided by this subfraction[32, 37].

The capacity of HDL_3 particles to remove oxidized lipids from

other lipoproteins and cellular membranes is greater than HDL_2 particles[38]. However, diabetes may lead to alterations in HDL composition and function, decreasing the beneficial properties of HDL[39]. Even though the quantity of HDL_3 did not differ between groups, it is reasonable to hypothesize that the quality and functionality may differ[40-41].

Jiang *et al*[42] studied HDL components in 90 patients diagnosed as having stable coronary heart disease by high-resolution polyacrylamide gel electrophoresis (detected by Quantimetrix HDL Lipoprint system). HDL particle size can be determined with great accuracy, using this method. Similar to our study, they found that serum levels of small HDL were not associated with the proinflammatory marker hsCRP in their patient groups.

In this study, serum FRAP levels was significantly decreased in diabetic patients compared to healthy controls due to the utilization of antioxidants against oxidative stress. Similar observations were reported previously[15, 43]. A decrease in total antioxidant capacity among DM2 subjects could be attributed to increased oxidative stress as evidenced by the production of various reactive oxygen and nitrogen species in diabetic tissues, including the retina[44-46].

Although the NOx level for diabetic patients was high compared to controls, it did not reach a statistically significant level. There are, however, conflicting results in the literature[47]. Increased serum levels of NO estimated by measuring serum nitrite and nitrate have been found in diabetic patients[14, 44-48]. Several studies documented that DM2 patients had significantly higher levels of serum, plasma and vitreous NO than diabetic patients without diabetic retinopathy or non-diabetic controls[46-49].

There are some restrictions to point out concerning the current study.

First of all, the method used might have influenced HDL_3 values in the present study. Various HDL subfractionation methods exist but there is no accepted gold standard technique for the measurement of HDL particles[50]. Many of the discordant results in this area may come from the multitude of different methodologies used to quantify subclasses of lipoproteins.

Secondly, the number of patients was small and from a single center. It is known that dietary factors influence HDL_2 and HDL_3 levels[51]; similarly nitrite and nitrate intake in food can influence NOx levels.

To sum up, determination of HDL_3 cholesterol, in addition to total HDL cholesterol which may not predict the actual risk for diabetic retinopathy. Serum NOx was observed to be higher in diabetic participants and total antioxidant capacity level was low.

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