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Correlation between Ultrasonographic Findings of Lower Extremity Vascular Disease and Plaque Formation and Carotid Atherosclerosis in Type 2 Diabetes Mellitus

Haiyan Nan, Ran Feng, Guiling Zhang and Jingqin Liu*

Department of Endocrinology, No.1 Hospital of Baoding, Baoding, 071000, China

ABSTRACT

This study aimed to evaluate the correlation between the ultrasonographic findings of lower extremity vascular disease and plaque formation and the formation of carotid atherosclerosis in patients with type 2 diabetes mellitus (T2DM). In this study recorded patients who were admitted to the No.1 hospital of Baoding from August 2017 to January 2020. We continuously observed the comprehensive diabetes complications screening or blood glucose of 517 hospitalized patients with T2DM. 67 subjects were excluded due to incomplete physical examination and clinical parameters, lack of carotid lower extremity ultrasonography, and 450 subjects participated in the study. There were 268 male patients with an average age of 54.35±6.37 and 182 female patients with an average age of 56.67±7.19. 450 healthy people were enrolled for the control group. According to the experimental requirements, the subjects were divided into two groups: the control group (healthy people without T2DM, n=450); the T2DM group (patients meeting the T2DM diagnostic criteria of the World Health Organization and diagnosed as T2DM, n=450); the general data of the subjects were statistically analyzed; the lower extremity arteries of the subjects were examined by color Doppler ultrasonography, including measurement of intima-media thickness, atherosclerotic plaque and stenosis; western blot and RT-qpcr were used to detect the FGF23 protein and mRNA expression results of this study showed that in the left pop artery, the inner diameter of the T2DM group was smaller than that of the control group ($P<0.05$), while the peak flow velocity, blood flow and spectral width of the T2DM group were higher than those of the control group ($P<0.05$). In the left and right dorsal foot arteries, the inner diameter and blood flow of the T2DM group were lower than those of the control group ($P<0.05$). The peak flow velocity and spectral width of the T2DM group were higher than those of the control group ($P<0.05$). Compared with the control group, the FGF23 protein content and the F-IMT thickness in the T2DM group were increased ($P<0.05$). Compared with the control group, the proportion of type I, type II and type III in the T2DM group was higher than that in the control group ($P<0.05$), and the proportion of type 0 in the T2DM group was lower than that in the control group. With the increase of carotid stenosis, the expression of FGF23 mRNA increased. The expression of FGF23 mrna in type I carotid stenosis was higher than that in type 0 carotid stenosis. The expression of FGF23 mrna in type III carotid stenosis was the highest ($P<0.05$). According to our results, carotid or lower extremity atherosclerosis was associated with increased risk in patients with T2DM, and FGF23 protein expression of patients increased, providing accurate warning for cardiovascular abnormalities in patients with vascular disease and T2DM.

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Key words
T2DM, Ultrasound doppler, Carotid atherosclerosis, Vascular disease

INTRODUCTION

The main cause of morbidity and mortality in patients with type 2 diabetes mellitus (T2DM) is diabetic macrovascular disease (Nakashima *et al*., 2018). Accumulated epidemiological and clinical evidence shows that T2DM patients have a higher risk of macrovascular diseases, especially coronary heart disease, cerebrovascular disease and peripheral vascular disease (Chan *et al*., 2018). The incidence of peripheral artery disease of individuals with diabetes is 2 to 4 times higher than that of healthy people, and the amputation rate of lower limbs is increased by about 15 times (He *et al*., 2017). Therefore, early detection of atherosclerosis in diabetes is very important to reduce the risk of diabetic macrovascular complications. The clinical manifestations of atherosclerosis mainly occur in coronary artery, lower limb artery and carotid artery (Yetkin *et al*., 2018). Ultrasonography is a non-invasive method for examining peripheral arterial walls (such as carotid artery and lower limb artery), and provides measurement of intima-media

* Corresponding author: Liujingqin11@aliyun.com
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thickness (IMT) and plaque (Sultana *et al*., 2020). At present, the examination of carotid artery by ultrasound has been used as a reliable and non-invasive method to evaluate the atherosclerosis process in large population (Ong *et al*., 2017). Fibroblast growth factor 23 (FGF23) is a protein mainly expressed by bone cells and osteoblasts in bone, playing an important role in the regulation of mineral metabolism (calcium phosphate homeostasis) (Kondo *et al*., 2017). Studies have shown that FGF23 can induce the occurrence and development of atherosclerosis through its influence on vascular calcification and endothelial dysfunction (Svanteson *et al*., 2017). The purpose of this study is to explore the relationship between T2DM and the prevalence of carotid artery or lower limb atherosclerosis, and to determine whether ultrasonography of carotid artery and lower limb artery is a more useful method to screen the generalized atherosclerosis of T2DM.

MATERIALS AND METHODS

The study recorded patients who were admitted to the No.1 hospital of Baoding from August 2017 to January 2020. We continuously observed the comprehensive diabetes complications screening or blood glucose of 517 hospitalized patients with T2DM. 67 subjects were excluded due to incomplete physical examination and clinical parameters, lack of carotid lower extremity ultrasonography, and 450 subjects participated in the study. There were 268 male patients with an average age of 54.35±6.37 and 182 female patients of 56.67±7.19. Four hundred and fifty healthy people were enrolled for the control experiment. Inclusion criteria: no gender limitation; patients younger than 88 meeting the diagnostic criteria of World Health Organization for T2DM. Exclusion criteria: lower limbs suffering from vascular diseases; infectious diseases; patients with severe cardiovascular and cerebrovascular diseases; patients with severe liver or kidney damage; patients with severe mental illness. Medical ethics: The Medical Research Ethics Committee approved the study, and all patients signed written informed consent.

Somatometry

The subjects took off their shoes and weighed them in light clothes with an error of 0.05 kg, and then measured their height, waist circumference and hip circumference accurate to nearly half a centimeter. BMI is calculated as the measured weight (kg) divided by the square of the measured height (m). Waist-hip ratio is calculated as waist circumference divided by hip circumference.

Research design

All subjects were interviewed to obtain their history of hypertension, cardiovascular and cerebrovascular diseases, and their drinking and smoking habits. Each patient received Doppler ultrasonography of carotid artery and lower extremity artery. Carotid and lower limb arteries were scanned to assess the occurrence of plaque and IMT thickness. According to the results of ultrasound ultrasonography, the prevalence of coronary artery and carotid artery atherosclerosis in all diabetic patients was evaluated.

Ultrasonography

In this study, Acuson Sequoia 512 scanner with 5-13MHz linear array transducer (Siemens Healthineers, California, USA) was used for color Doppler ultrasonography of lower limb arteries, including IMT, atherosclerotic plaque and stenosis measurement. That is to say, the ultrasonography is performed by three experienced sonographers according to the standardized scheme using the machine Acuson Sequoia 512 with a 5-13 MHz probe. After two groups of subjects kept supine for 5 minutes, transducers were continuously placed on their necks and lower limbs to display vascular imaging and blood flow characteristics. IMT and atherosclerotic plaque were recorded at each location. The carotid arteries were examined bilaterally in the transverse and longitudinal directions of the common carotid artery, bifurcation, external carotid artery and internal carotid artery. The lower limb arteries were evaluated bilaterally at 7 locations: common femoral artery, deep femoral artery, superficial femoral artery, pop artery, anterior tibial artery, posterior tibial artery and peroneal artery. The shape of the spectrum was examined to determine the vessel diameter, peak velocity, blood flow and spectral width, and the artery stenosis was calculated by the following formula: stenosis = (vessel diameter-vessel effective diameter)/vessel diameter× 100%. The stenosis was classified into four grades: type 0, type I, type II and type III.

Western blot and real-time quantitative PCR analysis of FGF23 expression

On the night before the experiment, the subjects were forbidden to eat after 10:00 pm, then their blood was drawn from vein at 7:00 am. The content of FGF23 in the subjects was determined by western blot analysis after optimizing the standard of serum samples. Then, the supernatant was collected and subjected to SDS-PAGE. After that, the protein was transferred to polyvinylidene fluoride (PVDF) membrane, and then blocked with 1% bovine serum albumin (BSA) at room temperature for one hour. After one hour reaction at 37°C, the reaction mixture was developed with goat anti-human IgG (Fc

specific)-horseradish peroxidase conjugate diluted at 1: 5,000: 2 mol/L H2SO4 (50µl per well) was added. The wells were washed with PBS-T three times between each intermediate step. GAPDH was used as an internal reference. Colorimetric results were recorded by Odyssey chemiluminescence instrument. ImageJ software was used for grey analysis to detect the expression level of target protein. All samples were determined in triplicate, and the values were expressed as mean ±S.D. cDNA was sythesized using the high capacity cDNA reverse transcription kit and qPCR analysis was performed with PerfeCTa SYBR Green Fastmix in the 7900HT fast real-time PCR system. Primers for qRT-PCR were as follows: FGF23: Fw: 5'-TTGGATCGTATCACTTCAGC-3' and Rev: 5'-TGCTTCGGTGACAGGTAG-3'. Rat GAPDH: Fw: 5'-GCAACTCCCATTCTTCCACC-30 and Rev: 5'-CATACCAGGAAATGAGCTTCACAA-3'.

Statistical analysis

All the data in this study were processed by SPSS20.0 statistical analysis software (IBM Company, USA). The measurement data was expressed by mean ±standard deviation. The comparison between groups was made by one-way analysis of variance or repeated measures analysis of variance, and the pairwise comparison between groups was made by LSD-t test; the counting data was expressed by percentage (%), and the comparison between groups was analyzed by χ^2. *P<0.05* indicated statistically significant difference.

RESULTS

General data statistics of clinical patients

The study recorded the patients who visited the Department of Endocrinology of Provincial People's Hospital from August 2017 to January 2019, and finally 450 subjects participated in the experiment. Among them, there were 268 male patients with an average age of 54.35±6.37 and 182 female patients with an average age of 56.67±7.19. 550 healthy people were enrolled for the control experiment. There was no difference in general statistics among subjects participating the experiment (gender, age, body mass index, waist circumference) (*P>0.05*) (Table I).

Table I. Clinical general data statistics (n=450).

Item	Control group	T2DM group	χ^2/t value	P value
Gender (male/female)	249/201	268/182	0.372	0.318
Age	53.62±6.17	55.49±6.37	1.362	0.714
Body mass index (km/m^2)	25.11±2.06	25.78±1.69	1.374	0.155
Waist circumference (cm)	92.64±8.76	91.58±8.23	1.102	0.238

Doppler ultrasonography of left pop artery

Table II shows dopper ultrasonography of left pop artery of lever limbs, left dorsalis pedis artery, and right dorsalis pedis artery. For left pop artery compared with the control group, the blood vessel diameter in T2DM group decreased (*P<0.05*), while the peak velocity, blood flow and spectral width in T2DM group increased (*P<0.05*). For dorsalis pedis artery it was found that the blood vessel diameter and blood flow in T2DM group were smaller than those in control group (*P<0.05*). Compared with the control group, the peak flow velocity and spectral width in T2DM group increased (*P<0.05*). For right dorsalis pedis artery compared with the control group, the blood vessel diameter and blood flow in T2DM group decreased (*P<0.05*), and the peak flow velocity and spectral width in T2DM group increased (*P<0.05*).

Western blot analysis of FGF23 expression

According to the western blot of the FGF23 protein content of both groups, the FGF23 protein content and the F-IMT thickness in T2DM group increased compared with the control group (*P<0.05*) (Fig. 1, Table II).

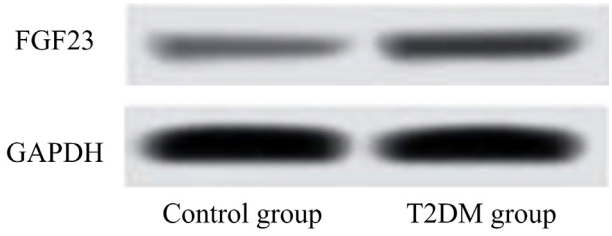

Fig. 1. FGF23 protein expression.

Relationship between T2DM and carotid stenosis

According to the classification definition, the carotid stenosis in both groups can be divided into four grades: type 0, type I, type II and type III. The proportion of type I, type II and type III in T2DM group was higher than that in control group (*P<0.05*), while the proportion of type 0 in T2DM group was lower than that in control group (P<0.05) (Table III).

Table II. Comparison of Doppler ultrasonography, FGF23 protein expression, and F-IMT thickness (n=450).

Group		Control group	T2DM group	t value	P value
Doppler ultrasonography	Blood vessel diameter	5.87±0.23	5.14±0.15	5.632	0.001
	Peak flow velocity	0.49±0.02	0.68±0.13	4.157	0.001
	Blood flow	12.47±1.68	14.42±2.47	6.447	0.008
	Spectral width	4.28±0.65	5.97±0.74	5.886	0.001
Doppler ultrasonography of left dorsalis pedis artery	Blood vessel diameter	1.86±0.25	1.29±0.13	4.528	0.007
	Peak flow velocity	0.41±0.03	0.51±0.02	6.397	0.005
	Blood flow	1.32±0.18	0.69±0.05	4.174	0.003
	Spectral width	4.35±1.05	6.07±1.14	5.226	0.003
Doppler ultrasonography of right dorsalis pedis artery	Blood vessel diameter	1.87±0.37	1.19±0.21	4.127	0.003
	Peak flow velocity	0.39±0.02	0.67±0.12	5.268	0.004
	Blood flow	1.56±0.25	0.67±0.13	6.337	0.006
	Spectral width	4.08±1.02	6.01±1.24	4.291	0.003
FGF23		1.23±0.26	2.97±0.35	5.268	0.001
F-IMT (mm)		0.69±0.15	0.86±0.23	6.322	0.001

Table III. T2DM carotid stenosis (n=450).

Group	Type 0	Type I	Type II	Type III
Control group	360(80.00%)	69(15.33%)	20 (4.44%)	1 (0.22%)
T2DM group	14 (3.11%)	342(76.00%)	73(16.23%)	21(4.67%)
χ^2 value	5.368	6.114	6.861	5.229
P value	0.001	0.001	0.001	0.001

Correlation between carotid stenosis and FGF23 mRNA expression

With the increase of carotid stenosis, the expression of FGF23 mRNA increased (r=0.231, p=0.0264). The expression of FGF23 mRNA in type I carotid stenosis was higher than that in type 0 carotid stenosis, and the expression of FGF23 mRNA in type III carotid stenosis was the highest (*P<0.05*) (Fig. 2).

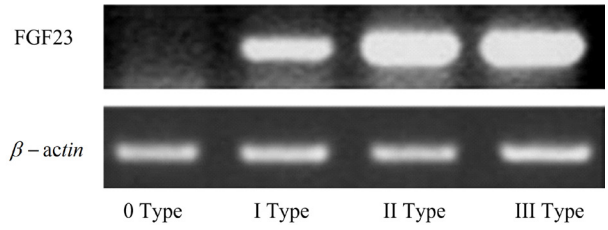

Fig. 2. FGF23 mRNA expression.

DISCUSSION

Carotid artery and lower limb vascular disease is one of the most serious complications of diabetes, and there is no clear pathological mechanism. Regardless of numerous factors of arteriosclerosis, the increase of diabetes incidence may be due to the increase of blood sugar and oxidative allergy and the production of free radicals which leads to the dysfunction of vascular tissue multicellular function. The dysfunction strengthens the interaction between leukocytes and endothelial cells. The oxidation reaction and glycosylation of low density lipoprotein and a series of chain reactions caused by the deposition of advanced glycosylation end products (Nolan and Prentki, 2019; Zheng *et al*., 2018; Cuenza *et al*., 2017). The continuous hyperglycemia of diabetes accelerates the process of advanced glycation end products, improves the formation ability of advanced glycation end products, and promotes the aggregation of advanced glycation end products in patients' tissues, the proliferation of vascular smooth muscle cells, the thickening or protrusion of vascular walls and the reduction of vascular elasticity (Abdellatif *et al*., 2017). The main pathological changes of vascular diseases in neck and lower limbs of diabetic patients are IMT thickening, vascular lumen narrowing, wall compliance reduction, formation of irregular atherosclerotic plaque, leading to further narrowing of lumen in intima, and then secondary thrombosis and even vascular occlusion (Yang *et al*., 2019; Okur *et al*., 2017). Atherosclerotic disease of lower limbs is the main manifestation of peripheral arterial disease, which represents systemic atherosclerosis

involving peripheral blood vessels. So it is related to the increased risk of cardiovascular disease (Jindal *et al*., 2018). Diabetes is an established risk factor for lower extremity atherosclerosis. Compared with patients without diabetes, patients with diabetes have worse prognosis of lower extremity atherosclerosis. They usually have faster progress of lower extremity atherosclerosis and wider range of vascular system disease, and are more susceptible to stenosis and occlusion (Filippou *et al*., 2017).

In the study, vascular stenosis was classified into four grades: type 0, type I, type II and type III. Compared with the control group, the proportion of type I, type II and type III in T2DM group increased, while type 0 in T2DM group decreased, indicating that the vascular disease in T2DM group were more serious than those in healthy control group. For diabetic patients, lower extremity atherosclerosis is usually not detected clinically in the early stage due to the common complications of neuropathy. It is not detected until the symptoms get worse and ulcer or gangrene occurs, usually in the end stage, and eventually leads to amputation (Estevez-Garcia *et al*., 2018). Therefore, early diagnosis and treatment of lower extremity atherosclerosis in patients with diabetes requires the index of early lower limb atherosclerosis, which will help to avoid amputation and improve patients' living quality. This study showed that serum FGF23 level in T2DM patients was higher than that in control group. Our analysis showed that the increase of serum FGF23 protein expression level was an independent risk factor of lower limb atherosclerosis, which was positively correlated with F-IMT. The accumulated clinical evidence shows that there is a relationship between serum FGF23 level and atherosclerosis, which is also supported by our previous research results. It has been proved that FGF23 in human coronary endothelial cells promotes oxidative stress and induces the release of nitric oxide, and its stimulating effect on the production of reactive oxygen species is offset by the increase of degradation of reactive oxygen species and inhibition of the bioavailability of nitric oxide, which leads to endothelial dysfunction and stimulates vascular calcification, and finally leads to atherosclerosis in diabetes (Szpera-Gozdziewicz *et al*., 2018). In this study, serum FGF23 level also showed independent and positive correlation with F-IMT. As IMT can be used as an index of early atherosclerosis, our results showed that serum FGF23 level should be used to indicate early clinical atherosclerosis. Ultrasonography is a non-invasive method to examine peripheral arterial walls (such as carotid artery and lower limb artery), and provides IMT and plaque measurement. At present, the ultrasonography of carotid artery has been used as a reliable and non-invasive method to evaluate the atherosclerosis process in large population (Memon, 2017; Nadim *et al*., 2018). In the study, the conditions of left pop arteries of lower limbs of two groups of subjects were compared. The blood vessel diameter in T2DM group decreased, while the peak velocity, blood flow and spectral width in T2DM group increased compared with the control group. With the increase of carotid stenosis, FGF23 mRNA expression increased. FGF23 mRNA expression in type I carotid stenosis was higher than that in type 0 carotid stenosis, and FGF23 mRNA expression in type III carotid stenosis was the highest. The clear and optimistic association between FGF23 level and F-IMT was one of the new points in this research. As IMT can be used as a primary atherosclerosis measure, our findings suggest that serum levels of FGF23 can be used to indicate primary clinical atherosclerosis. Ultrasound is a non-invasive procedure to test peripheral arterial walls (such as the artery of the carotid artery and the artery of the lower extremity) and provides assessments of IMT and plaque. With rising carotid stenosis, FGF23 mRNA expression also improved. In our study, we have shown that it offers IMT and plaque measurements and that the use of ultrasound to assess the diameter of the carotid artery can be useful in the early detection and prevention of serious complications in patients with type 2 diabetes.

To sum up, carotid artery or lower limb atherosclerosis was related to the increased risk of T2DM patients, and the expression of FGF23 protein in T2DM patients increased, providing accurate early warning for delaying vascular diseases and cardiovascular abnormalities in T2DM patients.

CONCLUSION

To conclude, propofol has a protective effect on myocardial ischemia-reperfusion injury in type 2 diabetic patients by improving cardiac function, increasing serum NO, Bcl-2, and SOD, and decreasing serum ET-l, cTnT, and IL- 1p, IL-6, TNF-a, BAX, LDA, CK, MAD.

Statement of conflicts of interest

The authors have declared no conflict of interest.

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