

CORRELATIONS BETWEEN DYSGLYCEMIA, MARKERS OF OXIDATIVE STRESS AND INFLAMMATION IN DIABETIC FOOT PATIENTS

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Abstract

Impaired glucose metabolism, dyslipidemia and chronic inflammation are present in diabetes mellitus. Ulceration of the foot in diabetes is common and disabling and frequently leads to amputation of the leg. The aim of this study was to assess the relationship between fasting plasma glucose (FPG), postprandial plasma glucose (PPG), plasma lipids and inflammation markers in diabetic foot patients.

Hospitalised diabetic foot patients (n=12), diagnosed with diabetes mellitus type 2, for less than 6 years, who haven't yet received insulin as treatment, were included. Spectrophotometric and HPLC methods were used to assess plasma studied parameters. Significant correlations (r with values between 0.81 and 0.94, for p<0.05) were calculated between LDL (low-density lipoprotein) cholesterol and: haptoglobin, ceruloplasmin, lipid peroxides, FPG, uric acid, glycosylated hemoglobin (HbA_{1c}), plasma dicarbonyls and fructosamine. Atherogenic index was positively correlated (r with values between 0.74 and 0.88, for p<0.05) with: haptoglobin, ceruloplasmin, lipid peroxides, leukocytes, HbA_{1c}, triglycerides. The leukocytes were correlated with acute phase proteins (r=0.82 with C-reactive protein and r=0.81 with haptoglobin). PPG didn't correlate with any of the above markers. Glucose metabolism was revealed by the average values: HbA_{1c} =10.14%, FPG=144mg/dL, dicarbonyls=0.49μmol/L and fructosamine 256.17 μmol/L. This pilot study underlines the strong correlations between precursors of AGE (advanced glycation end-products), dyslipidemia markers and acute phase proteins in a group of type 2 diabetic foot patients with incorrect glycaemic control and duration of diabetes for less than six years. Atherogenic index correlated with all the pathogenic markers in diabetes mellitus demonstrates the strong link between atherosclerosis and the diabetic foot complication.

Rezumat

Diabetul zaharat se caracterizează prin anomalii în metabolismul glucozei, dislipidemie și inflamație cronică. Ulcerația din piciorul diabetic este frecventă și invalidantă și conduce deseori la amputații. Scopul prezentului studiu a constatat în determinarea corelațiilor dintre glicemia *à jeun*, glicemia postprandială, lipidele plasmatică și markerii de inflamație la pacienții cu picior diabetic.

Au fost luați în studiu doisprezece pacienți internați pentru picior diabetic, având diabet zaharat tip 2 de mai puțin de șase ani și care nu au început tratamentul cu insulină. Au fost utilizate metode HPLC și spectrofotometrice pentru evaluarea parametrilor studiați. Au fost calculate corelații semnificative statistice (r cu valori între 0,81 și 0,94, pentru $p < 0.05$) între LDL colesterol (LDL - lipoproteine cu densitate scăzută) și următorii compuși: haptoglobină, ceruloplasmină, peroxizi lipidici, glicemie *à jeun*, acid uric, hemoglobină glicozilată (HbA_{1c}), dicarbonilii plasmatici, fructozamină. Indexul aterogenic a fost corelat pozitiv (r cu valori între 0,74 și 0,88, pentru $p < 0.05$) cu: haptoglobina, ceruloplasmina, peroxizii lipidici, leucocitele, HbA_{1c} , trigliceridele. Leucocitele au fost corelate cu proteinele de fază acută ($r=0.82$ pentru corelația cu proteina C reactivă și $r=0.81$ pentru relația cu haptoglobina). Glicemia postprandială nu s-a corelat cu nici unul dintre markerii de mai sus. Metabolismul glucozei a fost relevat de valorile medii ale: $HbA_{1c} = 10.14\%$, glicemia *à jeun* = 144mg/dL , dicarbonilii plasmatici = $0.49\mu\text{mol/L}$ și fructozamina plasmatică $256.17\mu\text{mol/L}$. Acest studiu pilot subliniază corelațiile semnificative dintre precursorii AGE (produși finali de glicare avansată), markerii dislipidemiei și proteinele de fază acută la un grup de pacienți cu diabet zaharat tip 2, de mai puțin de șase ani, având picior diabetic și un control deficitar al glicemiei. Indexul aterogenic s-a corelat cu toți markerii de patogeneză din diabetul zaharat și demonstrează legătura strânsă dintre ateroscleroză și piciorul diabetic.

Keywords: diabetic foot, inflammation, dysglycaemia, dyslipidemia, AGE (advanced glycation end-products)

Introduction

Ulceration of the foot in diabetes is common and disabling and frequently leads to amputation of the leg [1]. Diabetic foot ulcers occur as a result of various factors. Such factors include mechanical changes in the conformation of the bony architecture of the foot, peripheral neuropathy and atherosclerotic peripheral arterial disease, all of which occur with higher frequency and intensity in the diabetic population. Non-enzymatic glycosylation predisposes ligaments to stiffness. Neuropathy causes loss of protective sensation and loss of coordination of muscle groups in the foot and leg, both of which increasing mechanical stress during ambulation [2].

Taking into account the classification of diabetic complications, peripheral neuropathy is considered a microvascular complication, while atherosclerotic peripheral arterial disease is a macrovascular complication. Microvascular and macrovascular complications are mainly or partly dependent on dysglycemia, which has two components: chronic sustained hyperglycemia and acute glycemic fluctuations from peaks to nadirs. Both components lead to diabetes complications through two main mechanisms: excessive protein glycation and activation of oxidative stress. A few years ago, these two mechanisms were unified in an elegant theory that suggested that the glycemic disorders observed in diabetic patients result in an activation of oxidative stress with an overproduction of superoxide by the

mitochondrial electron-transfer chain. This activation in turn produces a cascade of such deleterious metabolic events as: enhanced polyol activity, increased formation of advanced glycation end products, activation of protein kinase C and nuclear factor κ B, and increased hexosamine pathway flux [3].

Inflammation and dyslipidemia are also important pathogenic mechanisms in diabetes mellitus. The state of chronic inflammation typical of obesity and type 2 diabetes occurs at metabolically relevant sites, such as the liver, muscle, and most interestingly, adipose tissues. Recent years have registered a critical progress in this respect by the identification of several downstream mediators and signalling pathways, which provide the crosstalk between inflammatory and metabolic signalling. These include the discovery of c-Jun N-terminal kinase (JNK) and I κ B kinase (IKK β) as critical regulators of insulin action activated by TNF- α (tumor necrosis factor α) and other inflammatory and stress signals, and the identification of potential targets [4].

Currently, glycosylated haemoglobin (HbA_{1c}) is considered the “gold standard” of glycemic control and is regarded as the key factor in reducing the risk of diabetes-related complications [5]. Hanefeld et al. demonstrated in the Diabetes Intervention Study that postprandial hyperglycemia was a better predictor of subsequent myocardial infarction and mortality than fasting hyperglycemia [6]. Post-prandial hyperglycemia is defined as a plasma glucose level exceeding 140mg/dL. Development of post-prandial hyperglycemia coincides with an impairment or absence of the first-phase insulin response, a decrease in insulin sensitivity in the peripheral tissues and decreased suppression of hepatic glucose output after meals due to insulin deficiency. Post-prandial hyperglycemia is also one of the earliest abnormalities of glucose homeostasis associated with type 2 diabetes, and worsens - progressing to fasting hyperglycemia - as the condition progresses [7]. To conclude, the pathophysiology of diabetes complications can be considered the result of two major deleterious metabolic alterations (excessive glycation and generation of oxidative stress) that are activated by three main glycemic disorders: hyperglycemia both at fasting and during postprandial periods and acute glucose fluctuations [29,30].

The aim of this study was to evaluate the relationships between fasting plasma glucose (FPG), postprandial glycemia (PPG), glycosylated hemoglobin HbA_{1c} on one side and the markers of oxidative stress, carbonylic stress and inflammation on the other side, in diabetic foot patients.

Materials and methods

Study subjects

Twelve hospitalised diabetic foot patients, aged between 45-75, diagnosed with diabetes mellitus between four and six years, were enrolled. They were recruited from The Cantacuzino Hospital, Bucharest, Romania and the exclusion criteria were: hemodialysis treatment, drug or alcohol abuse, use of vitamin, mineral, magnesium, lipoic acid supplements in the previous month. The severity of neuropathy and vascular disease were assessed by a diabetologist.

Blood samples were collected into 10 mL vacutaine tubes, containing heparine, from a peripheral vein after 12 hours of fasting and drugs break. For postprandial plasma glucose (PPG) a blood sample was taken 2 hours after lunch. The study protocol was approved by the Ethical Committee of "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania and a written informed consent was obtained from each study participant.

Routine blood tests including glycemia, total cholesterol, HDL-cholesterol (HDL – high density lipoprotein), triglycerides (TG) were analysed in the laboratory of the Cantacuzino Hospital, Bucharest, Romania (Roche reactivities, Germany). Total analytical variability, expressed as coefficient of variation (CV) was 5%, 5%, 23.6%, and 6.54% respectively. LDL-cholesterol (LDL – low density lipoprotein) was calculated according to the Friedewald equation [8]. Glycated hemoglobin HbA_{1c} was measured using HPLC cation exchange column and fructosamine was measured by the nitroblue tetrazolium colorimetric assay adapted for use in 96-well plates (Fruc, Roche Diagnostics, Germany). Haptoglobin and C-reactive protein (CRP) were assayed nephelometrically. Plasma uric acid was determined using a commercial kit (Human Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden Germany). Total plasma protein concentration was determined by Biuret method.

At the "Carol Davila" University, Faculty of Medicine, at the Biochemistry Department, blood samples were analyzed for oxidative and carbonylic stress markers, using spectrophotometric methods.

The d-ROM (determinable reactive oxygen metabolites) were measured using a commercial kit (Pharmalab d-ROMs, Parma, Italy). The peroxides present in plasma can produce radicals by a Fenton-like reaction, in the presence of Fe²⁺ ions. These radicals are chemically trapped by a fenolic derivative (N,N-diethyl-p-phenyldiamine) which is then itself transformed into a red-colored radical which absorbs slightly at 512 nm.

The intensity of the colour produced is proportional to the concentration of peroxides and is calibrated against known concentrations of tert-butyl hydroperoxide.

Total plasmatic malondialdehyde (MDA) was analysed by pretreating plasma samples with thiobarbituric acid in ortho-phosphoric acid containing butyl-hydroxytoluene as antioxidant. The pink coloured product was separated by HPLC using reverse phase LiChrospher RP C18 (Alltech, Deerfield IL), methanol/KH₂PO₄ 10mM (40/60 v/v) as mobile phase and detection at 532 nm [9].

Glutathione peroxidase (GPx) and Cu/Zn superoxide dismutase (SOD) were respectively measured using the RANSEL and RANSOD kits from Randox Laboratories, Antrim, UK.

Catalase (CAT) activity was adapted from literature [10]. In short, 200 µL of erythrocyte lysate was added up to 1300 µL of sodium phosphate buffer (50 mmol/L, pH 7). Hydrogen peroxide (750 µL) was added to a final concentration 13mmol/L and its decomposition monitored at 240 nm for 30 seconds at 37°C. Enzymatic activity was expressed as Kat/g hemoglobin where Kat is defined as Ln(initial absorption/ final absorption)/time interval (CV 5%).

Glutathion S-transferase (GST) activity was measured according to the literature [11]. In short, 100 µL of erythrocyte lysate were added to sodium phosphate buffer (100 mmol/L, pH 6.5) containing 0.1% Triton-X100, 2mmol/L reduced glutathione (Sigma Aldrich Chemical Co., St. Louis, Mo, USA). After addition of 1 mmol/L CDNB (1-chloro-2,4-dinitrobenzene, Fluka Chemika, USA) the formation of conjugated product, 1-chloro-2,4-dinitrobenzene-S-glutathione was monitored at 340 nm for 3 minutes at 37°C. Enzyme activity was calculated from its extinction coefficient, 9.6mM⁻¹cm⁻¹. One unit of GST is defined as 1 µmol of conjugate formed per minute on the above-described assay conditions (CV = 3%).

The plasma ceruloplasmin level determination is based on the oxidize activity of the protein towards p-phenylenediamine [12]. The dicarbonyls assay is based on their reaction with Girard's reagent T [[carboxymethyl)trimethyl-ammonium chloride hydrazide] at 294nm. Glioxal was used for the etalonation curve [13].

Data were analyzed using Statistic and Excel software. Differences between groups were assessed by Student's t-test. The relationship between the various parameters was assessed by correlation, stepwise multiple regression. Two-tailed p-values (p<0.05) were considered as statistically significant.

Results and discussion

The characteristics of the healthy subjects and diabetic patients are shown in table I, the measured markers of oxidative and carbonylic stress are illustrated in table II and the strongest correlations (r 0.74-0.91, $p < 0.05$) between the studied parameters are presented in table III.

Table I
Clinical characteristics of the healthy subjects and diabetic patients

Parameter	Controls n=20	Diabetic patients n=12	p value (controls vs. diabetic patients)
Age	64 $\pm$ 4.09	65.28 $\pm$ 8.7	ns
Duration of diabetes (years)	-	4.33 $\pm$ 1.63	-
Body mass index (BMI) (kg/m ²)	24.9 $\pm$ 1.2	27.84 $\pm$ 5.15	$p < 0.05$
HDL cholesterol (mg/dL)	59 $\pm$ 6.8	36.66 $\pm$ 11.96	$p < 0.005$
LDL cholesterol (mg/dL)	136 $\pm$ 44.6	121.32 $\pm$ 52.51	$p < 0.05$
Tryglicerides (mg/dL)	100 $\pm$ 55.8	119.33 $\pm$ 74.36	ns
Plasma total cholesterol (mg/dL)	218 $\pm$ 51.2	180.0 $\pm$ 70.2	$p < 0.05$
Atherogenic index	3.6 $\pm$ 0.9	5.4 $\pm$ 0.5	$p < 0.005$
HbA _{1c} (%)	5.4 $\pm$ 0.05	10.14 $\pm$ 1.94	$p < 0.001$
FPG (fasting plasma glucose) (mg/dL)	87 $\pm$ 28.9	144.37 $\pm$ 36.44	$p < 0.01$
PPG (postprandial plasma glucose) (mg/dL)	128 $\pm$ 64	216.25 $\pm$ 83.67	$p < 0.05$
Uric acid (mg/dL)	4.4 $\pm$ 1.98	5.34 $\pm$ 2.08	$p < 0.03$

ns= not significant

Table II

The measured markers of oxidative and carbonylic stress

Parameter	Controls n=20	Diabetic patients n=12	p value (controls vs. diabetic patients)
MDA mmol/L	0.41±0.12	0.64±0.16	p<0.04
dROM mM Trolox	2.37±0.9	3.82±1.05	p<0.005
CRP mg/dL	0.05±0.01	2.55 ± 2.09	p<0.05
Haptoglobin mg/dL	115.8±23.7	236.37±58.04	p<0.005
Ceruloplasmin (mg/dL)	25.94±9.8	36.75±9.55	p<0.05
Leukocytes	5500 ±2300	7687.5±2814	p<0.05
SOD U /g Hb	820±189	1233 ±288	p<0.03
GST U /g Hb	3.6 ±0.9	5.4±0.05	p<0.005
Dicarbonyls micromol/g proteins	0.30 ±0.10	0.49± 0.16	p<0.02
Fructosamine micromol /L	224.5±112.3	279.05 ±146.5	p<0.05

Table IIIThe correlations ($r = 0.74-0.91$, $p<0.05$) between the studied parameters

	dROM	Leukocytes	Uric acid	HbA1c	Duration	Fructosamine	Cholesterol	TG	LDL	Atherogenic index	Dicarbonyls
CRP		0.82									
haptoglobin							0.79	0.91	0.84	0.88	
ceruloplasmin	0.91						0.89	0.86	0.94	0.74	
dROM						0.87	0.81			0.82	
leukocytes										0.74	
FPG				0.67			0.85		0.86		
BMI			0.82		-98						
uric acid							0.89		0.84		
HbA1c								0.78	0.74	0.75	
dicarbonyls							0.93		0.88		
fructosamine							0.81		0.83		
cholesterol								0.87	0.98		0.93
HDL											
TG									0.89	0.79	
LDL											0.88
MDA							0.78		0.78	0.74	0.78

PPG was excluded from this table because it hasn't any important (r higher than 0.60) correlation with any other parameter that was taken into study.

It is known that diabetic complications in target organs arise from chronic elevations of glucose. The pathogenic effect of high glucose, in concert with fatty acids is mediated to a significant extent via increased production of reactive oxygen species (ROS) and subsequent oxidative stress [14]. Oxidative stress elicits systemic inflammation, promotes endothelial dysfunction, impairs pancreatic β cell insulin secretion and interferes with glucose disposal in peripheral tissues, thereby accelerating the progression of type 2 diabetes [15].

Postprandial and general glucose fluctuations play a major role in activating oxidative stress, leading to the endothelial dysfunction, one of the mechanisms responsible for vascular complications [16]. Monnier L. *et al.* reported that the risks for myocardial infarction and micro-vascular complications were diminished by 14% and 37%, respectively, for each 1% reduction in A_{1c} [16].

The relative contribution of PPG for the HbA_{1c} value varies. It is highest (more than 70%) in well-controlled diabetic patients who have HbA_{1c} levels of $\sim 6.5\%$ and lowest in incorrectly controlled diabetic patients (with HbA_{1c} levels $>8\%$), when the FPG level predominates. Thus, is better to use a triad model of diabetes management in which all three parameters - HbA_{1c} , PPG, and FPG levels - are considered to be interrelated and therapeutic targets could potentially optimize glycemic control [7].

In this study, in diabetic foot patients, HbA_{1c} was positively correlated with the plasma values for LDL cholesterol, for triglycerides and for atherogenic index. It is known that the lipid anomaly in diabetes is the combination of low HDL-cholesterol, high small dense LDL and hypertriglyceridemia [17]. Non-enzymatic glycation of apolipoproteins impairs lipoprotein clearance, augmenting dyslipidemia. Plasma triglycerides above normal are associated with the formation of the more atherogenic, small, dense LDL and they also have proatherogenic effects by promoting a procoagulant state, being associated with enhanced factor VII activity [18]. So, the above calculated correlations may explain the association between high values of HbA_{1c} and vascular complications, in diabetic foot patients. Eight from the twelve studied patients (66.6%) had micro and macro-vascular complications, which is an important figure, considering the duration of diabetes in the studied patients (less than 6 years).

At present, there is no doubt that excessive levels of glucose at fasting and during postprandial periods activate the glycation process, which can be investigated as a whole by measuring the HbA_{1c} levels. In addition to

hyperglycemia at fasting, acute or sustained hyperglycemia over postprandial periods and more generally acute glucose fluctuations around the mean glucose value activate oxidative stress [16].

Hyperglycemia and insulin resistance are independent risk factors for cardiovascular disease (CVD). Postprandial glycaemic "spikes" adversely affect vascular structure and function *via* multiple mechanisms including oxidative stress, inflammation, low-density lipoprotein oxidation, protein glycation and procoagulant activity [19].

In our study there wasn't any important correlation (with statistical significance) between PPG and markers of oxidative stress. A negative correlation ($r = -0.63$ and $p < 0.05$) was calculated between SOD activity and HbA_{1c}. This value is explained by the non-enzymatic glycation of the enzyme. GST and GPx activities were weak correlated with PPG ($r = 0.55$ and $r = 0.59$, respectively) and even more weak with FPG ($r = 0.32$ and $r = 0.33$, respectively). On a larger group these correlations would become important.

However, plasma lipids (LDL cholesterol, total cholesterol) were strongly correlated (r between 0.74 and 0.78) with plasma lipid peroxides (measured as MDA) and with markers of carbonylic stress (plasma dicarbonyls, fructosamine). The correlation between plasma MDA and dicarbonyls underlines the importance of lipoxidation in AGE formation. It is known that dicarbonyls are the major precursor of advanced glycation end-products (AGE) implicated in the development of diabetic complications. AGE proteins and AGE lipids are products of both glycooxidation and lipoxidation [20]. It is interesting to notice that although all the patients took statins and the average values for cholesterol and triglycerides are considered normal, the levels of lipid peroxides and dicarbonyls are above the control's levels and correlated ($r = 0.78$).

Hyperuricemia has been found to be associated with obesity and insulin resistance, and consequently with type 2 diabetes. We calculated a positive correlation ($r = 0.82$, $p < 0.05$) between the body mass index (BMI) and plasma uric acid in diabetic foot patients. In the last years, it was demonstrated that uric acid induces endothelial dysfunction and vascular inflammation, decreases endothelial nitric oxide production and becomes prooxidant in the vascular environment of diabetic patients (the urate redox shuttle) [21]. In turn, hyperuricemia leads to an increased risk of atherosclerosis. In consensus with the last statement we found a positive correlation between plasma uric acid and cholesterol (total and LDL).

There has been a recent explosion of interest regarding that chronic low-grade inflammation and the activation of the innate immune system are

closely involved in the pathogenesis of type 2 diabetes. It was demonstrated that markers of inflammation predict or/and are associated with type 2 diabetes and that inflammation is involved in the pathogenesis of atherosclerosis, a common feature of type 2 diabetes [22].

In this study, in diabetic foot patients, the increased values for the acute phase proteins, CRP, haptoglobin and ceruloplasmin reflect the presence of inflammation. The acute-phase proteins are mostly synthesized in the liver, and their production is stimulated by cytokines. The high values of these inflammatory markers are mainly secondary to obesity or act as surrogate markers of hypoadiponectinemia. Ceruloplasmin and haptoglobin known as antioxidants may be prooxidants in diabetic patients. An increase in serum ceruloplasmin in type 2 diabetes could generate an excess of oxidized LDL, which causes atherosclerosis [23]. The prooxidant site was localized in a region containing His⁴²⁶ (histidine). These observations support the hypothesis that copper bound at specific sites on protein surfaces can cause oxidative damage to macromolecules in their environment [24]. Ceruloplasmin could also cause vascular injury by generating free radicals, such as hydrogen peroxide, in the course of oxidization of serum homocysteine [25]. The positive correlation between plasma dROM and ceruloplasmin ($r=0.919$, $p<0.05$) is based on the fact that inflammation increases both lipid peroxides and acute phase protein levels.

A recent cross-sectional study demonstrated that acute foot ulcers and their severity are associated with a marked up-regulation of acute-phase proteins, cytokines and chemokines independently of the concomitant infection. An ideal therapy for diabetic foot ulcer should both suppress excessive inflammation while enhancing healing [26].

In this study, acute reactive proteins (haptoglobin and ceruloplasmin) are correlated with LDL cholesterol and atherogenic index and also with the level of leukocytes. It is known that there are three haptoglobin phenotypes and the 1-1 form is a better inhibitor of haemoglobin-induced lipid peroxidation than the 2-2 form. It seems that the 2-2 phenotype increases the risk for atherosclerosis in diabetes because it is less effective in blocking the pro-oxidant activity of glycated haemoglobins [27].

Because the acute-phase response and cytokinemia are so closely related to insulin resistance, the relationship with hyperglycemia is not unexpected. Lowering of blood glucose levels in type 2 diabetic patients is accompanied by reduced levels of inflammation markers [28].

Conclusions

Understanding the links between pathogenic mechanisms (dyslipidemia, dysglycemia, oxidative stress and inflammation) will have important implications for the design of novel therapies to reduce and delay complications development in diabetes mellitus, including diabetic foot.

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