In Vitro and Clinical Investigations in Lower Extremity Artery Disease with a Special Focus on Diabetes Mellitus

Ph.D. dissertation

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2018

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List of abbreviations

AI: aggregation index

ABI: ankle/brachial index

ACEi: angiotensin-converting enzyme inhibitor

cAMP: cyclic adenosine monophosphate phosphodiesterase

DPA: dorsal pedal artery

DRP: diabetic retinopathy

CAD: coronary artery disease

CLI: critical limb ischemia

CV: cardiovascular

CVD: cerebrovascular disease

DM: diabetes mellitus

EI: elongation index

EI_{max}: maximal EI at infinite shear rate

Hct: hematocrit

IC: intermittent claudication

LEAD: lower extremity artery disease

LORCA: Laser-assisted Optical Rotational Cell Analyzer

PAD: peripheral arterial disease

PPS: pentosan polysulfate sodium

PTA: posterior tibial artery

PV: plasma viscosity

PVP: polyvinylpyrrolidone

RBC: red blood cell

S.E.M: standard error of mean

$SS_{1/2}$: shear stress required for half of EI_{max}

$tcpO_2$: transcutaneous partial tissue oxygen pressure

$t_{1/2:}$ aggregation half time by LORCA

T2DM: type 2 diabetes mellitus

6MWT: 6-minute walk test

WBV: whole blood viscosity

WIFI: wound, ischemia, foot infection

γ: disaggregation threshold shear rate

Prologue

Peripheral arterial disease (PAD) is the third leading cause of atherosclerotic cardiovascular morbidity, following coronary and cerebrovascular diseases. More than 200 million people suffer from PAD worldwide, of whom nearly 40 million live in Europe [1]. At least 50% of all PAD patients are asymptomatic; therefore, many of them lack proper diagnosis and treatment in time [2, 3]. Both asymptomatic and symptomatic PAD patients have high risk for cardiovascular death [4], thus early identification, prevention and treatment could reduce mortality [5]. PAD appears usually over the age of 50, increases exponentially with age, the rate can reach 20% by the age of 80 years. Based on data from recent decades, additional increase of prevalence and incidence can be expected as a result of global aging, increase in the number of smokers, diabetics, hypertensive and over-weighted patients as well as growing sedentary lifestyle. Lower extremity artery disease (LEAD) has several different presentations according to Fontaine classification, which is a commonly used clinical staging system from grades I (asymptomatic) to IV (ulceration and gangrene). The symptomatic disease is only the tip of the iceberg, most patients are asymptomatic due to walking disabilities e.g. heart disease, musculoskeletal disorders or reduced pain sensitivity caused by diabetic polyneuropathy but low ankle/brachial index (ABI) or absence of pulse can be experienced. These subgroups are called “masked LEAD”. In most studies the proportion of symptomatic LEAD is 1:3 to 1:5 of all LEAD patients [6]. Even with a similar extent and level of disease progression, symptoms may vary from one to another. Intermittent claudication (IC) is defined as reproducible discomfort in a muscle induced by exercise training and relieved with rest. Although calf muscles are most often affected, muscles in the thigh or buttock may be affected by proximal arterial obstruction. LEAD is regarded as a heterogeneous group of patients ranging from patients with IC to patients with critical limb ischemia (CLI). CLI refers to a state of arterial insufficiency that reduces distal perfusion pressure, therefore nutrition and microcirculation of tissues are severely disturbed. This condition includes ischemic rest pain of the lower extremity, non-healing wounds, or tissue necrosis (gangrene) and is strongly associated with a high burden of amputation and limited life expectancy mostly in patients with diabetes mellitus. Risk factors contributing to LEAD are the same as those for atherosclerosis. The main risk factors include smoking, the risk increases with the smoking intensity. The most common risk factor for LEAD is diabetes mellitus (DM), the risk increases with the duration of the disease. In patients with diabetes, LEAD is associated with earlier large vessel involvement and atherosclerosis affects

mostly the distal arteries, and causes distal symmetrical neuropathy. The incidence of CLI is 10 to 20 times higher in patients with DM, moreover they have a 5-fold higher risk of amputation compared to non-diabetics primarily due to increased sensory neuropathy and decreased resistance to infection. The American Diabetes Association recommends PAD screening of diabetic patients measuring ABI regularly [7]. Dyslipidemia is a significant contributor to PAD [8], while hypertension is the strongest predictor of incidence of LEAD, including acute and chronic limb ischemia and limb-threatening ischemia [2].

The Framingham Study and other epidemiological investigations, e.g. the Edinburgh Artery Study, the Monica Project, the Honolulu Heart Program, the Physicians' Health Study, the Caerphilly Study and the Speedwell Study have reported that besides conventional cardiovascular risk factors, such as age, male sex, hypertension, smoking, dyslipidemia, and diabetes mellitus hemorheological parameters are primary and independent cardiovascular risk factors e.g. hematocrit, fibrinogen and viscosity [3, 9-15]. Several clinical studies have described an association between hemorheological parameters - including hematocrit, plasma (PV) and whole blood viscosity (WBV), reduced erythrocyte deformability and increased aggregation - and macro or microangiopathies in diabetes. These hemorheological alterations called “non-classic” cardiovascular risk factors may have a remarkable effect on the whole vascular system causing development of wide range of cardiovascular, cerebrovascular and peripheral arterial diseases [12, 16]. Previous studies demonstrated that PV and WBV are increased due to hyperglycemia leading to more pronounced erythrocyte aggregability and impaired red blood cell (RBC) elongation. In small blood vessels, where cells must deform to pass through narrow capillaries, deformability and aggregation of RBCs play an important role [16-18].

Clinical importance of hemorheological alterations in peripheral arterial disease

Hemorheological alterations can be associated with the early stages of atherosclerosis [19]. Atherosclerotic lesions usually tend to occur at special location of the arterial wall, at the bifurcations and the distal wall of arterial curvatures. In the Edinburg Artery Study fibrinogen level, plasma (PV) and whole blood viscosity (WBV) correlated with carotid intima/media thickness on multivariate analysis [3]. At physiological circumstances in the large vessels blood flow is determined by hemodynamic factors but in the distal narrow arteries hemorheological parameters may have an important role [20]. The alterations of hemorheological parameters in diabetes mellitus and peripheral arterial disease have been described by several studies in the last few decades, which can be considered as potential risk factors of cardiovascular diseases. Impairment of these factors may have a role in tissue hypoperfusion and disturbances of microcirculation [9, 10, 12].

Hematocrit

Hematocrit (Hct) is a widely used hemorheological parameter in the daily clinical practice describing the percentage of the cellular fraction of the whole blood. Hct and its determinants affect most of the other hemorheological parameters. Increase in Hct leads to a linear increase in oxygen binding capacity and an exponential increase in blood viscosity. Elevated Hct has been well studied as a cardiovascular risk factor, it is associated with increased cardiovascular mortality and morbidity [16, 17].

Plasma viscosity

It has been demonstrated that plasma viscosity plays an important role in the regulation of vascular tone and influences blood flow characteristics. Due to the axial migration of erythrocytes plasma viscosity is relevant in defining wall shear stress. The characteristic of plasma viscosity is determined by the amount of plasma proteins, such as fibrinogen, globulins, and triglycerides [16, 17].

Whole blood viscosity

Whole blood viscosity is mostly influenced by the hematocrit, plasma viscosity, red blood cell aggregation (at low shear rates) and deformability (at high shear rates). Higher WBV leads to flow resistance increase, while flow rate decreases. The increased stress affects the blood vessels facilitating vascular remodeling and accelerated atherosclerosis [17, 20, 21]. In PAD patients elevated fibrinogen levels, increased plasma and whole blood viscosity and Hct and higher hemoglobin values were observed [11, 22]. In patients with CLI similar PV was detected, while lower WBV and erythrocyte aggregability could be observed compared to healthy volunteers.

Red blood cell aggregation

Erythrocytes have a tendency to form aggregates. The process is reversible, occurs at low shear conditions or at stasis. RBC aggregation depends on hematocrit, intrinsic cell characteristics (also known as red blood cell aggregability) and the quantity of plasma proteins and lipids. Elevated RBC aggregation can be found in vascular diseases and chronic clinical conditions such as myeloma multiplex, autoimmune disorders, sepsis and malignant conditions [23]. In a recent study RBC aggregation was significantly elevated and plasma fibrinogen concentration was increased in PAD patients compared to the control group [24].

Red blood cell deformability

The ability of the erythrocyte to deform is a cornerstone of the cell passing through the narrow capillaries. The deformability of red blood cell is determined by several factors such as cell shape, membrane surface area, the internal viscosity of the cell and the membrane viscoelasticity [25, 26]. Decreased RBC deformability is associated with several factors, in some cases genetic origin can be found in the background (hemoglobinopathies, enzyme deficiencies of RBC metabolism, or modifications of cell membrane proteins), or parasite infection (e.g. malaria), in other cases mechanical trauma (artificial heart valve, extracorporeal circulation), oxidative damages (ischemia/reperfusion injury, mitochondrial leakage, activated leukocytes, transfusion causing iron overload) can lead to impaired deformability [27]. The study group demonstrated significantly different RBC deformability in PAD patients with complication of diabetes mellitus [28].

Focus and aim of the studies

In vitro hemorheological effects of parenteral agents used in peripheral arterial diseases

In daily clinical practice several drugs have been used as vasoactive agents with the lack of evidence. The effect of certain drugs has not been addressed in prospective, multicenter, randomized clinical trials. In those cases when endovascular or surgical revascularization can not be performed due to high cardiovascular risk or severe diabetic angiopathy, the question may arise whether vasoactive infusion therapy is justified. Based on these considerations the aim of our study was to evaluate the effect of alprostadil, iloprost, pentoxifylline, pentosan polysulfate and sulodexide on the hemorheological parameters in blood samples collected from healthy male volunteers.

Lower limb ischemia and micro-rheological alterations in patients with diabetic retinopathy

In Hungary around 800,000 people suffer from diabetes mellitus. Half of the patients die of cardiovascular diseases and 10-20% due to end stage renal failure. Neuropathy can be diagnosed in 50% of the patients and visual impairment or blindness develops in 10% of this population. In our country approx. 4,000 amputations of the lower limbs are caused by complications of diabetes annually. In our clinical study the primary goal was to screen the prevalence of lower extremity artery disease in diabetic patients who were regularly checked for retinopathy but systematic screening for PAD had previously not been performed. Our secondary aim was to find association between the measures of lower limb ischemia (6-minute walk test, $tcpO_2$) and hemorheological variables.

In vitro hemorheological effects of parenteral agents used in peripheral arterial diseases

Introduction

The management of PAD include pain relieving, wound care and infection control, prevention of atherosclerosis progression, improving quality of life, preventing limb loss, prolonging survival and control of cardiovascular risk factors, smoking cessation, regular supervised and home-based exercise training. Current pharmacological regimen contains antiplatelet agent in symptomatic LEAD and statin protecting against cardiovascular morbidity and mortality, angiotensin-converting enzyme inhibitors (ACEi), moreover, strict glycemic control is mandatory in diabetic patients [29, 30].

Nowadays several vasoactive drugs are available in PAD treatment. However, their impact on hemorheological factors is not well defined and most of these agents are lacking evidence on the improvement of morbidity and mortality in PAD; even their mechanism of action needs further clarification. Cilostazol, pentoxifylline, pentosan polysulfate and naftidrofuryl are the most recently used vasoactive drugs. Based on the patient's condition invasive procedure should be recommended: percutaneous transluminal angioplasty, stent implantation end/or surgical bypasses. In critical limb ischemia, revascularization is the primary therapeutic procedure to alleviate symptoms and salvage the limb [6], but it is frequently not feasible at all, and therefore alternative treatments should be considered, e.g. hemodilution, vasoactive drugs, intermittent pneumatic compression, electric nerve stimulation, or carbon dioxide enriched bath. Ernst et al. found that hemodilution significantly lowers hematocrit (Hct) and apparent whole blood viscosity (WBV), furthermore improves pain-free walking distance resulting in higher quality of life in patients with femoro-popliteal occlusion [31]. A meta-analysis reported significantly increased maximal walking distance with pentoxifylline [32]. Patients with CLI at high WIFI (wound, ischemia, foot infection) score with severe co-morbidities or limited options for revascularization may undergo primary amputation. Therapeutic angiogenesis or stem cell therapies may represent a novel approach to increase blood flow to ischemic tissues by the induction of a collateral network promising therapeutic possibility in the management of PAD.

Materials and methods

Blood samples collected from 19 non-smoker healthy male volunteers (mean age 27.2 ± 4.3 years) were used in the study. All volunteers gave their informed consent before blood donation. The study was approved by the Regional Ethics Committee of the University of Pecs, (licence number: 5075).

Blood sampling and sample preparation

Blood samples were collected from an antecubital vein after a 12- hour fast. Blood was taken into EDTA coated Vacutainer tubes with a 21-gauge butterfly infusion set according to the latest hemorheological guideline [33]. Drugs were added to the blood samples to reach the therapeutic serum concentration; saline solution was added to the control samples in order to eliminate any dilution-caused rheological alterations (10 µl saline solution was added to 3990 µl blood). Every sample was incubated for 1 hour at 37 °C on rollerbed. The following drugs were investigated in our study:

Iloprost is a synthetic analogue of prostacyclin (prostaglandin I). It is a potent vasodilator and inhibits platelet activation in vivo and both platelet aggregation and platelet and leukocyte adhesion in vitro [34]. 12.5 µl iloprost (20 µg/ml, Bayer Schering Pharma) was added to 3987.5 µl blood sample calculated to be equivalent with 62.5 pg/ml plasma concentration (National Institute of Pharmacy and Nutrition, Hungary).

Action mechanisms of alprostadil (prostaglandin E) include peripheral vasodilation, improvement of microcirculation, and inhibition of platelet aggregation [35]. 6 µl alprostadil (20 µg/ml, Gebro Pharma GmbH) was added to 4994 µl blood sample to reach 2.4 pg/ml plasma concentration [36].

Pentoxifylline is a xanthine derivative; it improves blood fluidity by reducing rigidity of red and white blood cells [37]. Furthermore, it decreases fibrinogen concentration, platelet adhesiveness and whole blood viscosity [38]. 10 µl pentoxifylline (20 mg/ml, Sanofi-Aventis) was dissolved in 3990 µl blood sample, the calculated plasma concentration was 1000 ng/ml [39].

Sulodexide is a highly purified mixture of glycosaminoglycans composed of heparan sulfate (80%) with affinity to antithrombin III and dermatan sulfate (20%) with affinity for heparin cofactor II [40]. 8 µl sulodexide (600 lipasemic units (LSU)/2 ml, Alfa Wassermann) was mixed with 3992 µl blood sample considered to be equal to 0.12 LSU/ml plasma concentration (National Institute for Quality- and Organizational Development in Healthcare and Medicines, Hungary).

Pentosan polysulfate sodium is a heparinoid with anticoagulant and fibrinolytic properties; it may also have lipid lowering and anti-inflammatory effects [41]. We dissolved 8 µl pentosan polysulphate sodium (100 mg/ml, bene-Arzneimittel GmbH) in 3992 µl blood, the calculated plasma concentration was 0.020 mg/ml (National Institute of Pharmacy and Nutrition, Hungary).

Hemorheological measurements

Hemorheological measurements were performed within 1 hour after incubation.

Hematocrit

Hematocrit (Hct) was measured by microhematocrit centrifuge (Haemofuge Heraeus Instr., Germany).

Plasma and whole blood viscosity

Plasma viscosity (PV) and whole blood viscosity (WBV) were measured by Hevimet 40 capillary viscometer (Hemorex Ltd., Budapest, Hungary) (Figure 1). Plasma was prepared by a 10-minute centrifugation of whole blood at 1500 g. 620 µl of sample was injected into the viscometer and let the flow out. The viscometer contained 40 diodes, the height of each diode from the baseline was known, so the shear stress and the hydrostatic pressure of the fluid could be calculated. PV and WBV values were interpolated at 90 s^{-1} shear rate in the study. Measurements were completed at 37 °C temperature. From the above mentioned data Hct/WBV ratio was calculated, which refers to the oxygen transport efficiency of blood [42].

Figure 1. Hevimet 40 capillary viscometer

Red blood cell aggregation

Red blood cell (RBC) aggregation was measured by Myrenne (MA-1 Aggregometer, Myrenne GmbH, Roetgen, Germany) [10, 12] and LORCA (Laser-assisted Optical Rotational Cell Analyzer; R&R Mechatronics, Hoorn, Netherlands) aggregometers [33].

Myrenne aggregometer applies the light transmission method of Schmid-Schönbein et al. through transparent cone-plate shearing instrument. Blood sample (30 μl) is sheared at 600 s^{-1} to disperse all pre-existing aggregates, then shear rate falls to zero (M mode) or to 3 s^{-1} (M1 mode). Measurements were established at room temperature (22±1 °C) [43]. Myrenne provides two dimensionless indices at room temperature (M, aggregation at stasis; and M1, aggregation at low shear), both values are increased with enhanced red blood cell aggregation.

LORCA aggregometer (Laser-assisted Optical Rotational Cell Analyzer; R&R Mechatronics, Hoorn, Netherlands) detects the laser back-scattering generated by erythrocytes. 1 ml of oxygenated blood is injected into a gap between the static inner cylinder ("bob") and the rotating outer cylinder ("cup") which creates a simple shear flow. The blood samples are characterized by the aggregation index (AI), half time ($t_{1/2}$) – the time required for the half of the maximal aggregation, and threshold shear rate (γ), this represents the smallest shear stress required for complete disaggregation. The temperature was kept at 37 °C [44]. Backscattering

of laser light suddenly increases (shape recovery phase) then decreases causing RBC aggregation drawing "syllectogram". AI is calculated from the areas A and B of the diagram (AI=A/A+B) during the first 10 seconds of the measurement (Figure 2).

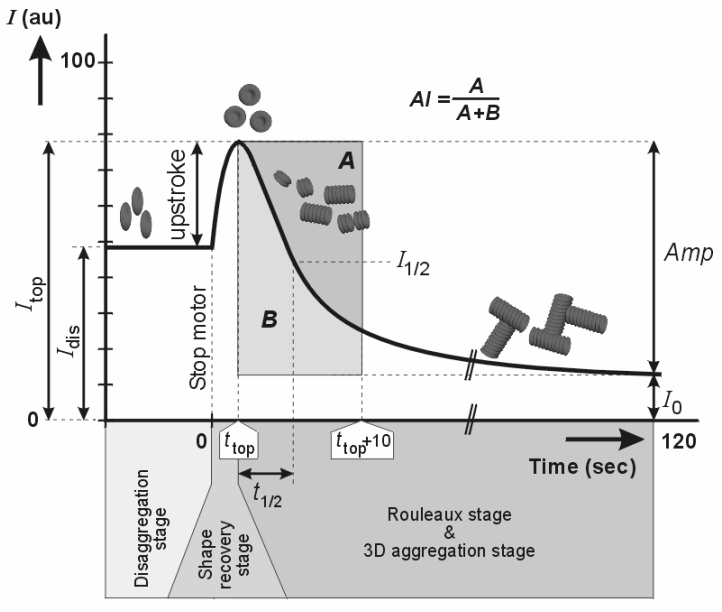

Figure 2. RBC aggregation parameters obtained using a syllectogram as determined by LORCA (Handbook of Hemorheology and Hemodynamics, IOS Press, Amsterdam, pp. 250-256, 2007).

Red blood cell deformability

LORCA ektacytometer was used for measuring erythrocyte deformability (Figure 3).

20 µl of blood was suspended in 4 ml high viscosity (29.43 mPas) polyvinylpyrrolidone (PVP) solution. RBCs were deformed by 9 different shear stresses from 30 Pa to 0.3 Pa.

Figure 3. LORCA ektacytometer

RBC deformability is characterized by the elongation index (EI) from the two diameters of the ellipsoid diffraction pattern as (A-B)/(A+B). Deformability results were analyzed by the Lineweaver-Burke nonlinear equation calculating the maximal elongation index (EI_{max}) at infinite shear, and the shear stress value ($SS_{1/2}$) which is required for the half of this maximal elongation.

Statistical analysis

Data are shown as means ± SD. Differences were evaluated by Student's one-sample t-test after using the Kolmogorov–Smirnov test to check the normality of the data distribution and f-test to determine variance equality. Differences were considered significant at $p<0.05$.

Results

Our results are summarized in Tables 1 and 2.

Iloprost did not show any significant effect on plasma and apparent whole blood viscosity, furthermore we did not find any significant alteration in RBC elongation and aggregation.

Alprostadil did not have any statistically significant effect on plasma and apparent whole blood viscosity values and we did not find any influence on aggregation parameters. We observed statistically significant increase in the elongation index values at 0.53 and 0.95 Pa shear stresses ($p < 0.05$).

Incubation with pentoxifylline had no significant effect on plasma and apparent whole blood viscosity and RBC aggregation. Elongation indices at 0.53 - 5.33 Pa shear stresses showed significant decrease ($p < 0.05$).

Sulodexide caused significantly lower apparent whole blood viscosity ($p < 0.05$), and consequently significantly higher hematocrit/WBV ratio ($p < 0.05$). LORCA AI was not different between control and sulodexide incubation. The calculated EI_{max} was significantly higher in sulodexide samples ($p < 0.05$).

Incubation with pentosan polysulfate sodium resulted in significantly higher apparent whole blood viscosity ($p < 0.05$) and significantly lower Hct/WBV blood viscosity ratio ($p < 0.05$); moreover, we found significantly ($p < 0.05$) higher aggregation parameters (AI, $t_{1/2}$). Elongation indices measured by LORCA ektacytometer did not show any significant changes.

Table 1. Effects of iloprost, alprostadil, pentoxifylline, sulodexide and pentosan polysulfate on macrorheological parameters and RBC aggregation.

Parameter	Control	Iloprost	Alprostadil	Pentoxi-fylline	Sulodexide	Pentosan polysulfate
Hct (%)	46.57±2.43	46.77±2.71	46.64±2.70	46.8±1.54	47.00±2.35	46.73±1.95
WBV (mPas)	4.64±0.42	4.67±0.43	4.64±0.50	4.62±0.37	4.50±0.42*	4.77±0.37*
PV (mPas)	1.26±0.07	1.28±0.07	1.27±0.05	1.26±0.07	1.26±0.04	1.22±0.08
Hct/WBV (1/Pas)	10.06±0.63	10.09±0.56	10.16±0.80	10.18±0.70	10.47±0.61*	9.71±0.67*
AI	59.35±6.89	58.99±6.47	60.15±6.87	61.06±8.70	60.06±5.16	61.35±9.02*
$T_{1/2}$ (s)	2.67±0.89	2.71±0.88	2.57±0.9	2.49±1.09	2.55±0.64	2.48±1.11*
γ (s^{-1})	79.47±24.16	77.5±27.89	80.00±30.25	79.32±27.25	70.00±12.5	82.5±33.41

*=significant difference between drug treated and controlled samples ($p<0.05$).

Table 2. Effects of iloprost, alprostadil, pentoxifylline, sulodexide and pentosan polysulfate on RBC deformability.

Shear stress	Control	Iloprost	Alprostadil	Pentoxi-fylline	Sulodexide	Pentosan polysulfate
30 Pa	0.63±0.01	0.63±0.01	0.63±0.01	0.62±0.07	0.63±0.01	0.63±0.008
16.87 Pa	0.60±0.01	0.60±0.01	0.60±0.01	0.59±0.009	0.60±0.01	0.60±0.01
9.49 Pa	0.56±0.01	0.56±0.01	0.56±0.01	0.55±0.01	0.56±0.01	0.55±0.012
5.33 Pa	0.50±0.01	0.50±0.01	0.50±0.02	0.49±0.01*	0.50±0.02	0.49±0.013
3 Pa	0.43±0.02	0.43±0.02	0.43±0.02	0.41±0.01*	0.43±0.02	0.42±0.02
1.69 Pa	0.34±0.18	0.34±0.02	0.34±0.05	0.32±0.02*	0.34±0.03	0.32±0.02
0.95 Pa	0.23±0.02	0.23±0.02	0.24±0.02*	0.21±0.02*	0.23±0.03	0.22±0.02
0.53 Pa	0.12±0.02	0.12±0.02	0.13±0.02*	0.10±0.02*	0.12±0.03	0.11±0.03
0.3 Pa	0.03±0.02	0.03±0.02	0.04±0.01	0.02±0.033	0.02±0.03	0.02±0.03
EI_{max}	0.673±0.006	0.672±0.005	0.672±0.007	0.674±0.009	0.676±0.008*	0.675±0.01

*= significant difference between drug treated and controlled samples ($p<0.05$).

Discussion

Revascularization procedures are not feasible for all symptomatic PAD patients, in these cases conservative therapeutic options could be considered, such as vasoactive drugs. In our current study the hemorheological effects of vasoactive drugs available as a parenteral agent in Hungary were investigated. These agents are administered in a venous line in the clinical practice, thus the role of first-path metabolism in the liver is attenuated and their effect on the red blood cells could be modeled in an *in vitro* manner. In cases of stenosed rigid proximal vessels and consequential maximal vasodilation distal to the lesion, blood flow velocity can be slower and temperature could be lower than the normal core temperature (especially in the acral parts); therefore vasodilative mechanisms could be insufficient and hemorheological factors could be more important. Rheological therapies were found to be effective in improving blood flow and pain-free walking distance in PAD patients [31].

Parenteral fluid administration results in hemodilution, which may improve hemorheological parameters. Parenteral fluid with a vasoactive drug content could be considered as improving macro- and microcirculation in those cases when endovascular procedure or surgical revascularization is not feasible. While the previous guidelines mentioned vasoactive drugs, the current ESC/ESVS guideline recommends antiplatelet and statin therapy, while vasoactive agents are not recommended because of the lack of evidence [6]. In the AHA/ACC guideline beyond the best medical preventive therapy cilostazol is mentioned with a class and level of evidence I/A, and pentoxifylline is not recommended, (III) [29]. According to the Hungarian PAD guideline cilostazol is recommended (I/A), naftidrofuryl should be considered (II/A) and pentoxyfilline may be considered (II/B) beyond the best medical treatment [45]. Parenteral agents are not recommended or mentioned at all in these guidelines.

A number of studies have shown that prostaglandin E1 and prostacyclin are more effective in the clinical outcome of PAD compared to other substances, e.g. pentoxifylline. Prostaglandin E1 and prostacyclin did not only show a reduction in peripheral vascular resistance but they are considered to have a significant range of other benefits in the treatment of vascular diseases [46]. Iloprost added to standard therapy significantly reduced whole blood viscosity and increased physical performance in patients with PAD [47]. In our in vitro study we did not find any significant hemorheological alterations with iloprost incubation in the measured parameters.

The anti-ischemic mechanisms of PGE in patients with PAD are complex, not limited only to a direct vasodilator action. This agent can inhibit the expression of adhesion molecules, platelet aggregation, monocyte and neutrophil function [35]. Our *in vitro* research showed a slightly but significantly improved RBC deformability at low and medium shear stresses which may improve microcirculation [25]. There is still a debate which of the two prostanoids is a more potent agent in PAD but we have insufficient amount and quality of data [48]. Several studies have been conducted to investigate the action of pentoxifylline in vitro and in vivo on hemorheological parameters. Pentoxifylline is a xanthine derivative and a nonspecific inhibitor of cAMP phosphodiesterases; it is still widely used in Hungary as a routine treatment of various cerebrovascular and peripheral vascular diseases characterized by a defective tissue perfusion [49, 50]. The therapeutic effect of pentoxifylline is considered mainly due to its ability to improve microvascular blood flow. The compound has been reported to increase flexibility of red blood cells and to decrease blood viscosity [51, 52]. Additionally, it was demonstrated that pentoxifylline reduces RBC aggregation [53]. Previously our team investigated the antioxidant

capacity of pentoxifylline in an *in vitro* mode. No significant activity could be detected at calculated therapeutic serum concentration, only at 100-times higher concentration [54]. Similarly, in this study we could not find any significant effects of pentoxifylline at therapeutic serum concentration on plasma and whole blood viscosity and RBC aggregation; furthermore, a slightly but significantly decreased deformability could be detected. Pentoxifylline's clinical efficacy is also questioned; neutral effect or slight improvement was reported in intermittent claudication [55].

Sulodexide has a number of effects on the structure and function of endothelial cells, and the intercellular matrix. According to the literature it helps protect or restore the integrity and permeability of endothelium against chemical, toxic or metabolic injury; inhibits aggregation and adhesion of platelets at the level of the vascular wall, reduces plasma fibrinogen concentrations, reduces plasminogen activator inhibitor-1 and increases tissue plasminogen activator as well as systemic fibrinolytic and thrombolytic activity [40]. Sulodexide alleviates the symptoms in chronic venous disease and accelerates the healing of venous leg ulcers; it improves intermittent claudication in patients with PAD and ameliorates kidney function in diabetic patients [5]. Authors reported significant reduction in serum viscosity, fibrinogen and triglyceride levels and elevation of high density lipoprotein in PAD patients [57]. The reduced Hct/WBV ratio was found as a mortality risk factor in a more recent investigation [42]. The incubation with sulodexide resulted in significantly lower apparent whole blood viscosity value and significantly higher hematocrit/WBV ratio. In *in vivo* observations WBV decrease could be associated with lower plasma viscosity and RBC aggregation, and improved RBC deformability, which could not be found at our measurements; therefore the causes of WBV reduction needs further clarification. Beneficial hemorheological effects could contribute to the advantageous findings regarding this drug in a previous study [58].

Pentosan polysulfate sodium (PPS) is a low molecular weight heparin-like compound still available in Hungary; it is a semi-synthetic heparin-like glycosaminoglycan. Although its exact mechanism of action is unknown, pentosan polysulfate may act as a buffer to control cell permeability by preventing irritating solutes from reaching cells coated with it. It has some anticoagulant and fibrinolytic effects. We found significantly higher whole blood viscosity at incubation with PPS *in vitro* and therefore significantly lower hematocrit/whole blood viscosity ratio; moreover, we detected significantly higher aggregation parameters and no alteration in the elongation index. In a recent study the therapeutic effectiveness of cilostazol and pentosan polysulphate were compared in PAD patients of grade Fontaine II. In both cases the pain-free

walking distance and the maximal walking distance increased significantly, there was no difference between the two groups [59].

Symptomatic PAD patients have a worse prognosis and frequently poorer quality of life than patients with coronary artery or cerebrovascular diseases. The low attention, the late diagnosis, the less intensively modified risk factors, the lack of lifestyle modifications and the limited number of evidence-based medical therapy can be revealed in the background of this unfavorable scenario. Our present study found that some of the vasoactive drugs can be beneficial on hemorheological parameters. Knowing this important mechanism can help us elaborate more effective treatment regimens.

The question may arise on the in vivo effectiveness of these agents. Therefore following this *in vitro* study, we performed a pilot *in vivo* study on the effects of parenteral agents. Alprostadil and sulodexide were investigated, hemorheological measurements (fibrinogen level, Hct, PV, WBV, RBC aggregation) and non-invasive arterial diagnostic procedures (ABI, $tcpO_2$, maximal and pain free walking distance) were performed in 15 patients on alprostadil and 13 patients on sulodexide. At baseline and after 1, 3, 6 months significant differences could not be found either in the hemorheological parameters or in the vascular measures, data were not published.

Study limitations

The study has an *in vitro* design with the general limitations of models. Under *in vivo* circumstances the presence of the vascular wall and the endogenous nitric oxide production could play an important part in the actions of a drug. Although first - path metabolism is supposed to be lower with intravenous administration, metabolic activity and modifications in the liver or within the vessel can modify the effect of a drug. The role of cellular interactions with the endothelium could not be addressed either. Venous samples were drawn from healthy subjects; the alterations might be more significant in the blood of PAD patients.

Conclusion

Beyond the previously described effects of the drugs involved in our study, we could find slight beneficial hemorheological alterations regarding sulodexide and alprostadil what could be important when vasodilator capacity becomes insufficient in peripheral arterial disease.

Lower limb ischemia and microrheological alterations in patients with diabetic retinopathy

Introduction

The prevalence of diabetes mellitus (DM) is increasing rapidly raising a huge burden on the healthcare system all over the world [60]. The complications of this disease (e.g. retinopathy, nephropathy, neuropathy, cardiopathy, and diabetic foot syndrome) can be attributed to macro- and microangiopathies. Diabetes mellitus is a major risk factor for the development of atherosclerosis. Although its severe consequences are well known, organ damages remain frequently undiscovered resulting in the high rate of end-stage disease. Retinopathy as a complication of microarteriopathy can be the first detectable subclinical lesion in diabetes which may reflect microcirculatory damages in other parts of the vascular system.

The association between diabetes mellitus and peripheral artery disease (PAD) has also been well established in several studies [8, 60]. PAD is two to four fold more frequent in diabetes mellitus and it starts 10 years earlier than in non-diabetic subjects [2]. It usually develops in both legs and progresses more rapidly resulting in multisegmental lesions. Revascularization procedures have poorer outcome and amputation is frequently needed. In diabetes PAD is often asymptomatic, therefore systematic screening should be performed [2, 8, 61, 62].

The diagnosis of PAD is routinely based on physical examination and Doppler-assisted peripheral blood pressure measurement. Hand-held Doppler is a valuable tool in classifying non-diabetic patients but it may give unreliable results in diabetes due to calcification of the calf arteries called Mönckeberg's media sclerosis [29]. In diabetes the calf arteries are frequently calcified, and thus the cuff pressure can be much higher than real intra-arterial pressure giving the impression of normal ankle-brachial index [2].

Other non-invasive vascular tests, e.g. toe pressure, $tcpO_2$ measurement or laser Doppler flowmetry for checking the regularity degree of skin blood flow should be considered but they are infrequently used in everyday clinical practice [30, 63]. Microcirculatory disorders in diabetes may not be attributed only to angiopathies but at least partially to hemorheological changes, like increased red blood cell aggregation and reduced red blood cell deformability. In a recent study correlation between whole blood viscosity and endothelial dysfunction was investigated in diabetes [64].

Methods

Participants

105 patients with type 2 DM were enrolled in the study, who attended regularly the out-patient clinic of the Department of Ophthalmology (mean age 64.64±9.01, 48 females and 56 males). To test age-dependent and independent changes two control groups were recruited.
42 non-smoking young individuals (mean age 25.52 ±3.32 years, 20 females and 22 males) performing regular physical exercise (minimum half an hour aerobic physical activity at least three times per week) and 35 age-matched non-diabetic volunteers (mean age 61.65±7.6 years, 21 females and 14 males) were involved who performed hiking at least once a week.
Co-morbidities, risk factors, medication were questioned and physical examination including palpation of peripheral arteries (dorsal pedal artery-DPA, posterior tibial artery-PTA, popliteal, femoral artery) were done.

Non-invasive arterial diagnostic procedures

Hand-held Doppler, ankle/brachial index

The commonly accepted method to investigate macrocirculation is the examination of peripheral pulses by hand and Doppler-assisted peripheral blood pressure measurements, which has a great value in classifying non-diabetic foot, but it can give unreliable results in case of diabetes.

The ankle pressures were measured by using hand-held Doppler ultrasound (MultiDoppy, 8 MHz, Medicad Ltd., Hungary, serial number: 141203) and a manual sphygmomanometer to measure systolic blood flow in posterior tibial and dorsal pedal artery of both legs as well as in the brachial artery of both arms following the same sequence of measurement. The cuff was placed around the ankle approximately 1 cm above the medial malleolus with parallel wrapping. To calculate the ankle-brachial index (ABI), the higher systolic blood pressure between both arms was used as the denominator while the higher pressure from the posterior tibial and dorsal pedal arteries at each ankle was considered as the numerator (Figure 4).

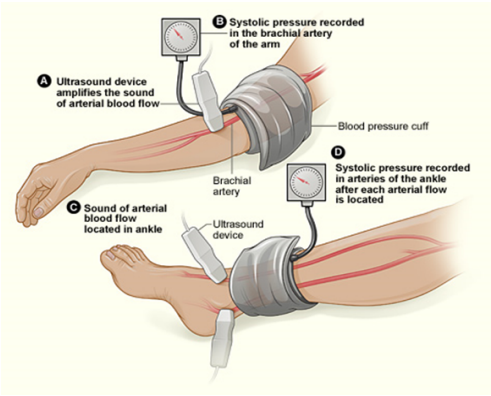

Figure 4. Hand-held Doppler measurement

Source: www.escardio.org/guidelines. 2017 ESC Guidelines on the Diagnosis and Treatment of Peripheral Arterial Diseases, in collaboration with ESVS. (European Heart Journal 2017; doi:10.1093/eurheartj/ehx095)

Transcutaneous tissue oxygen pressure

Measurement of transcutaneous partial oxygen tension ($tcpO_2$) is a noninvasive method detecting microcirculation and oxygen supply. It can give information on skin perfusion and ischemia, which can be used to define the degree of peripheral vascular disease and clinical progression of PAD. $TcpO_2$ can give the estimation of the partial pressure of oxygen on the skin surface using a noninvasive method [65]. $TcpO_2$ is dependent on oxygen uptake in the respiratory system, the oxygen transport of the blood and the general status of the circulatory system.

The electrode heats the underlying tissue causing maximal local vasodilation, which intensifies the blood perfusion, leading to increased oxygen pressure. The heat can dissolve the lipid structure in the epidermal layer, leading skin more permeable to gas diffusion. Nowadays, there are numerous possibilities for clinical applications, so this procedure has become a routine measurement in several clinical areas including: determination of peripheral tissue oxygenation, quantification of the degree of peripheral vascular disease, evaluation of revascularization procedures, determination of the optimum level of amputation and more recently it has been increasingly used as a screening tool to select candidates for hyperbaric oxygen therapy and predict non-responders to treatment.

In healthy subjects while breathing normobaric air the average $tcpO_2$ on the foot is >50 mmHg [66]. $TcpO_2$ values have a tendency to increase from the distal part of the body (foot) to proximal part (thigh), but some variability has been shown in other clinical researches [66, 67,

68]. Patients with critical limb ischemia almost always have a $tcpO_2$ value below 30 mmHg and in many cases less than 20 mmHg. Low oxygen tension can be caused by microvascular disease (e.g. diabetes mellitus), or oedema, inflammation, or vasoconstriction (e.g. cold exposure, pain, dehydration). Values of $tcpO_2$ need to be assessed in connection with the whole clinical picture. The transcutaneous oxygen pressure was measured with a two-channel oximeter (Tina TCM 4000, Radiometer, Copenhagen, Denmark) (Figure 5).

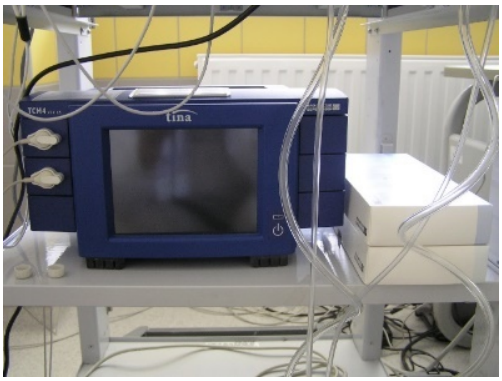

Figure 5. Oximeter (Tina TCM 4000, Radiometer, Copenhagen, Denmark).

Subjects could become acclimated for 15 minutes in a lying position at room temperature (22-24°C). The measured site of the skin was shaved and cleaned with alcohol. The self-adhesive fixation ring was filled with a contact liquid. The Clark electrode was placed to three different places on the body. A reference value was obtained by placing the electrode on the chest on the right side in the subclavicular region, thereafter the electrode was placed on the lateral part of the leg and on the dorsal part of the foot in the first intrametatarsal space - or if the patient underwent amputation on the most distal part of the leg - not over a visible vein, bony or tendon structure was chosen (Figure 6). Transcutaneous oximetry was performed at 44°C causing a maximum vasodilation in the Clark electrode contact site, so $tcpO_2$ depends only on the arteriolar partial pressure of oxygen. The electrodes were allowed to equilibrate until stable values were achieved [65]. During the functional test the patient was in a resting recumbent position for 15 minutes, which was followed by 5 minutes elevation and another 5 minutes hanging (stasis) of the leg.

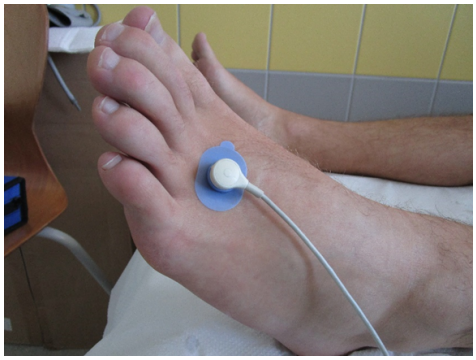

Figure 6. Measurement of transcutaneous partial oxygen tension

Calibrated tuning fork test

One of the main complications of diabetes mellitus is polyneuropathy responsible for more than half of all limb amputations [69]. Long asymptomatic latency period is part of the natural history. Screening for neuropathy is essential for the diagnosis, patient education, the provision of further impetus for optimization of glycemic control, and improving foot care for the reduction of lower-extremity complications [61, 70]. Screening for neuropathy is necessary in case of diabetes mellitus or for other metabolic reasons. Regular check-up is recommended in every diabetic patient annually [7]. Calibrated tuning fork test is easy to use, values below 4 refer to neuropathy. To examine sensory loss due to diabetic polyneuropathy Rydel-Seiffer calibrated tuning fork was used. 128 Hz tuning fork was applied on the same bony prominences bilaterally situated over the radius, ulna and on the dorsum of the first toe. The patient was asked to report the time at which vibration disappears [65, 71, 72].

6-minute walk test

An occasion of 6-minute walk test (6MWT) the patient can walk with at his own, maximal speed on 30 m long corridor. Pain free walking distance and maximal walking distance can be measured, the number of stopping, usage of any medical aids can give information about the walking capacity of the patient. Moreover, complaints can be provoked. The 6MWT was performed indoors along a straight corridor and walking course was 30 m in length according to the guideline of the American Thoracic Society [73].

Blood sampling, sample preparation

Blood samples were collected for hemorheological measurements by venipuncture after overnight fast. The blood specimen was collected in EDTA-coated tubes. Parameters were measured within 2 hours from blood withdrawal. Micro-rheological measurements (RBC aggregation and deformability) were performed according to the above mentioned methodological description (see page 13, 14), the same way as in our in vitro study.

Red blood cell aggregation and deformability

Erythrocyte aggregation and RBC elongations were determined by LORCA (Laser-assisted Optical Rotational Cell Analyzer; R&R Mechatronics, Hoorn, The Netherlands) ektacytometer.

Statistical analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) statistical software, version 11.0.1 for Windows. One-way repeated ANOVA statistical test and Bonferroni post-hoc test were used to evaluate differences between and within the groups after using Kolmogorov-Smirnov test to check normality of the data distribution. Data are shown as means ± standard error of mean (SEM). The level of significance was considered as p value <0.05. Pearson correlation coefficients were calculated to analyze relationships between continuous variables.

Ethics approval and consent to participants

The investigation has been performed in accordance with the Declaration of Helsinki and has been approved by the Regional Ethics Committee of the University of Pecs (number of investigation: 5121) and the Data Protection Service of the University of Pecs. In accordance with institutional guidelines written informed consent was obtained from all subjects before their participation.

Results

Epidemiological data

105 patients with DM, 35 age-matched non-diabetic patients, and 42 non-smoking healthy volunteers were recruited. No history of intermittent claudication, ischemic heart disease, polyneuropathy or retinopathy could be revealed in the control groups. 22.9% of the diabetic patients had PAD in the past medical history (e.g. intervention or surgery of the lower limb), 19% of them complained about claudication and almost 10% had ulceration. Epidemiological characteristics are summarized in Table 3.

Table 3: Characteristics of the study population.

	Diabetic patients (n=105)	**Non-diabetic patients** (n=35)	**Young volunteers** (n=42)
Mean age (yrs)	64.64±9.01	61.65±7.60	25.52 ±3.32
Gender (%)			
Male	53.4	60.0	47.6
Female	46.6	40.0	52.4
Mean duration of DM (yrs)	15.4	-	-
Type of DRP (%)			
Proliferative	23.8	-	-
Non-proliferative	76.2	-	-
Smoking habits (%)			
Current	12.4	2.4	-
No/Former	87.6	97.6	100
Hypertension (%)	96.2	53.0	-
Dyslipidemia (%)	65.7	16.6	-
Coronary artery disease (%)	29.5	-	-
Cerebrovascular disease (%)	11.4	-	-
PAD in the history (%)	22.9	-	-
Amputation (%)	6.7	-	-
Claudication (%)	19.0	-	-
Leg ulcer (%)	9.5	-	-

Young volunteers did not take any medication regularly. Drug treatment of diabetic patients and aged-matched non-diabetic subjects is summarized in Table 4.

Table 4. Medication of diabetic and age-matched non-diabetic groups.

	Diabetic patients (%)	**Non-diabetic patients** (%)
Oral antidiabetic drug (OAD)	37	-
Insulin	24	-
Insulin + OAD	39	-
ACEI/ARB	95	53
Statin	67	20
Antiplatelet	71	23
Oral anticoagulant	17	-

Non-invasive arterial diagnostic procedures

At physical examination, all control subjects (young volunteers and non-diabetic patients) had all pedal pulses present. 34% of the diabetic patients (n=36, 24 males, 12 females) had at least one non-palpable distal artery (DPA or/and PTA). In spite of antihypertensive therapy significantly higher blood pressure was observed both in the diabetic population and the age-matched non-diabetic group compared to the young persons (Table 5).

Table 5. Results of the systolic brachial and ankle blood pressures (mmHg) measured by hand-held Doppler.

	Diabetic patients	Non-diabetic patients	Young volunteers
Left arm	146.08±2.40[a]	141.45±3.05[a]	115.70±2.15
Right arm	148.41±2.08[a]	141.12±2.13[a]	117.81±3.20
Left DPA	143.09±5.08	139.21±3.23	126.43±2.48
Left PTA	143.04±4.93	143.44±3.53	132.92±2.62
Right DPA	142.30±5.60	137.65±4.07	128.37±2.50
Right PTA	142.35±5.31	142.50±4.22	133.24±2.45

a: significant difference compared to the young group ($p<0.001$).

The ABI of each young healthy volunteer was within the normal range. Most of the age-matched control subjects had normal (1.0-1.3) or borderline (0.9-1.0) ABI, only one patient had a moderately abnormal value. Less than half of the diabetic patients had normal ABI, more of them could be classified into the various abnormal ABI ranges (<0.9 or >1.3). Two patients had critically low ABI value (Table 6)**.**

Table 6. Distribution of ankle-brachial index in the study population.

ABI range	Diabetic patients (%)	Non-diabetic patients (%)	Young volunteers (%)
<0.4	1.90	-	-
0.4-0.7	9.52	-	-
0.7-0.9	12.38	2.86	-
0.9-1	15.24	57.14	-
1-1.4	46.67	40.00	100
>1.4	14.29	-	-

Transcutaneous partial tissue oxygen pressure was measured to detect lower limb ischemia. In the diabetic population, significantly lower $tcpO_2$ values were measured at every localization compared to the young volunteers and it was lower in the diabetic than in the non-diabetic group at the level of the leg. Age-matched controls had also lower $tcpO_2$ on the foot than the young population (Table 7).

Table 7. Results of $tcpO_2$ measurements.

Position of the electrode	**Diabetic patients** (mmHg)	**Non-diabetic patients** (mmHg)	**Young volunteers** (mmHg)
Chest	52.46±1.54 [a, b]	63.32±1.35	68.78±2.57
Leg at rest	46.81±1.59 [a, b]	55.35±3.42	60.02±1.92
Leg at elevation	43.13±1.50 [a]	50.93±3.56	51.24±2.45
Leg at stasis	58.11±1.58 [a, b]	68.00±2.76	66.05±2.22
Foot at rest	40.06±1.40 [a]	42.91±2.22 [a]	55.32±1.92
Foot at elevation	37.88±1.91 [a]	37.78±2.44 [a]	51.02±2.37
Foot at stasis	51.25±1.78 [a]	55.05±3.22 [a]	67.69±1.25

a: significant difference compared to the young group ($p<0.05$).
b: significant difference compared to the non-diabetic group ($p<0.05$).

Regarding the various $tcpO_2$ ranges (normal, borderline, decreased, severe ischemia), only a fifth of the diabetic patients had normal values and almost one fifth of them had abnormal results characteristic for the severe limb ischemia (Table 8).

Table 8. Distribution of the study population in the various $tcpO_2$ ranges (measured on the foot at rest).

$TcpO_2$ ranges	**Diabetic patients (%)**	**Non-diabetic patients (%)**	**Young volunteers (%)**
>50 mmHg	20.93	25.0	67.6
40-50 mmHg	30.23	53.12	27.0
30-40 mmHg	30.23	15.62	5.4
<30 mmHg	18.61	6.26	-

Sensing of vibration in diabetic patients was deteriorated compared to the other groups. 23 % of the diabetic population had low (<4) sensing of vibration. Age-matched control subjects had also lower sensing at the level of the toe than the young volunteers (Table 9).

Table 9. Results of the calibrated tuning fork test.

Localization	Diabetic patients	Non-diabetic patients	Young volunteers
Hallux	4.38±0.21 [a, b]	5.78±0.09 [a]	7.65±0.15
Proc. styl. radii	6.3±0.16 [a, b]	7.34±0.12	7.57±0.15
Proc. styl. ulnae	6.3±0.15 [a, b]	7.28±0.09	7.71±0.12

a: significant difference compared to the young group ($p<0.05$).
b: significant difference compared to the non-diabetic group ($p<0.05$).
The test was performed on both sides without a difference, values above represent the left side.

In the 6-minute walk test, maximal walking distance of diabetic, age-matched and young population was 275.22±13.01 (min. – max. 55 – 450), 410.51±6.53 (320 – 470) and 572.20±19.69 (378 – 890) meters, respectively; the difference was significant ($p<0.001$). In the diabetic population eight individuals could not complete the 6MWT due to painful ulcer on the leg or rheumatic problems, 19% of the diabetic patients had claudication during the test, while subjects in the other groups did not experience any complaints.

Hemorheological alterations

The results of RBC aggregation characterized by aggregation index, half-time for aggregation and disaggregation threshold shear rate are presented in Table 10. Aggregation index (AI) was significantly higher in the diabetic population and the age-matched group compared to the young volunteers ($p<0.05$); significant difference between the two patient groups could not be observed.

Table 10. Results of erythrocyte aggregation.

Aggregation parameters	Diabetic patients	Non-diabetic patients	Young volunteers
AI	67.38±0.70 [a]	64.32±11.43 [a]	59.09±1.10
$t_{½}$	1.79±0.07 [a]	1.83±0.091 [a]	2.74±0.15
γ	146.36±5.91 [a,b]	122.79±6.9 [a]	85.67±4.01

a: significant difference compared to the young group ($p<0.05$).
b: significant difference compared to the non-diabetic population ($p<0.05$).

Elongation index at each measured shear stress was significantly lower among diabetic patients compared to the non-diabetic group, and significant difference could be observed between the diabetic and the young groups at low and intermediate shear stresses (from the range 5.33 to 0.3 Pa). Significant difference could be found between the non-diabetic population and the young controls at high-intermediate and low shear stresses (range from 0.3 to 0.95 and from 3 to 9.49 Pa). The RBC deformability results were analyzed by the Lineweaver-Burke nonlinear equation calculating the theoretical maximal elongation index at infinite shear (EI_{max}). Although significant difference could not be found in the EI_{max}, the shear stress required for the half of this maximal elongation ($SS_{½}$) was significantly higher in the diabetic patients compared to the healthy volunteers and the elderly persons (Table 11).

Table 11. Results of RBC deformability

Shear stresses (Pa)	Diabetic patients	Non-diabetic patients	Young volunteers
30	0.617±0.001 [b]	0.625±0.0009	0.622±0.002
16.87	0.588±0.0013 [b]	0.597±0.0011	0.592±0.002
9.49	0.541±0.0014 [b]	0.550±0.0011 [a]	0.546±0.0021
5.33	0.482±0.0016 [a, b]	0.497±0.0013 [a]	0.489±0.0028
3	0.403±0.0018 [a, b]	0.423±0.0018 [a]	0.413±0.0039
1.69	0.308±0.0024 [a, b]	0.333±0.0025	0.321±0.0039
0.95	0.201±0.0027 [a, b]	0.230±0.0031 [a]	0.214±0.0049
0.53	0.096±0.0026 [a, b]	0.123±0.0035 [a]	0.103±0.0053
0.3	0.012±0.0031 [a, b]	0.037±0.0041 [a]	0.016±0.0014
EI_{max}	0.674±0.017	0.664±0.009	0.673±0.170
$SS_{1/2}$	2.59±0.82 [a, b]	1.91±0.28	2.16±0.42

a: significant difference compared to the young group ($p<0.05$).
b: significant difference compared to the non-diabetic group ($p<0.05$).

Relation of the walking distance to the circulatory and hemorheological variables

Within the diabetic population significant correlation could be revealed between the maximal walking distance and erythrocyte aggregation: higher AI, lower $t_{1/2}$ and higher γ were associated with shorter walking distance (r values: -0,363†, 0.249†, and -0.419‡, respectively; $^{\dagger}p<0.05$, $^{\ddagger}p<0.0001$) (Figure 7).

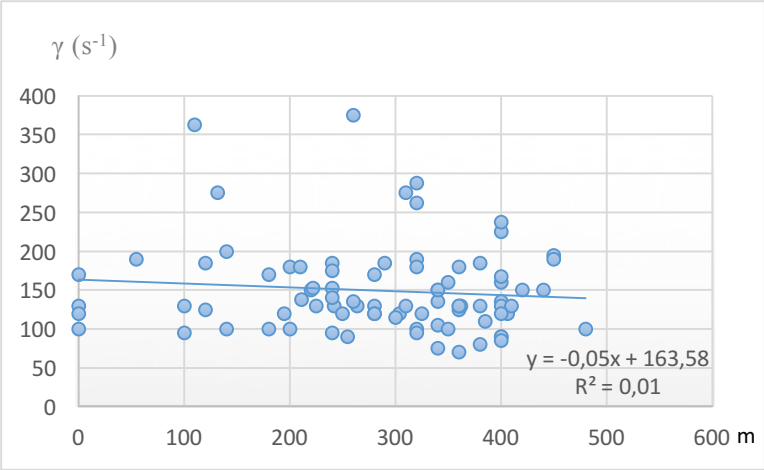

Figure 7. Correlation between the maximal walking distance and erythrocyte aggregation characterized by disaggregation shear rate threshold (γ).

In addition, RBC elongation characterized by EI_{max} had a positive correlation with the maximal walking distance (r=0.328, p<0.05). Ankle pressures (DPA, PTA) and $tcpO_2$ values on the leg and on the foot at stasis correlated slightly but significantly to the covered maximal walking distance (r values: 0.246, 0.251, 0.268, and 0.337, respectively; p<0.05) (Figure 8).

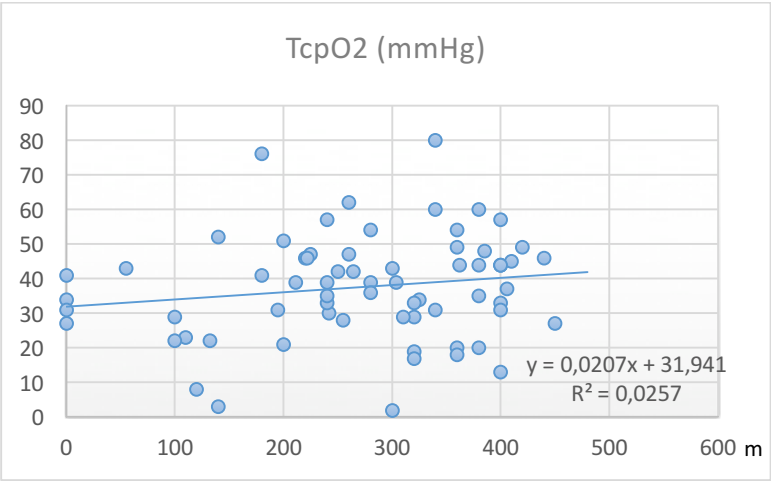

Figure 8. Correlation between the maximal walking distance and tcpO2 values at the stasis on the leg.

Discussion

The prevalence of lower extremity artery disease is high but largely underestimated, and significant differences could be found in population studies and clinical series. LEAD is two to fourfold more frequent in diabetes mellitus based on the literature, but true prevalence of LEAD in diabetic population is difficult to determine, because most patients are asymptomatic (partially due to polyneuropathy), and screening has not been performed uniformly [2]. Vascular abnormalities and hemorheological disturbances in diabetes impair the microcirculation provoking organic damages [74]. The presence of DM in LEAD patients increases the risk of adverse outcomes including progression to critical limb ischemia, amputation and death [6]. Kolossvary et al. presented that diabetes related incidence of major amputation was 7 times higher than in the general population and 15 times higher than in non-diabetic subjects. These data highlight the importance of screening and early detection of PAD [75]. PAD is an independent predictor for cardiovascular (CV) ischemic events not only in symptomatic but also in asymptomatic diseases. The high number of asymptomatic patients underlines the importance of vascular screening. Regular screening of diabetic patients for LEAD is recommended by several guidelines [2, 6, 76], but performed rarely.

In our study we aimed to search for lower extremity arterial disease in a regularly checked diabetic population. Two control groups were also studied to explore the age dependent and independent alterations. It is known that patient-reported symptoms underestimate LEAD prevalence [2, 6]. Based on the case history, LEAD had already been recognized in every fifth case in our study and 20% of the diabetic population had claudication. According to the literature and American Diabetes Association about 1 in 3 people with diabetes over the age of 50 have LEAD [6, 77].

Numerous non-invasive and invasive tests have been designed for screening and diagnosing LEAD in the clinical practice. Hand-held Doppler examination is a simple and cheap method to measure blood pressure of the lower extremities above the ankle. It has a high specificity (98%) and sensitivity (90%) to detect LEAD if the stenosis is more than 50% [2]. ABI is a good surrogate marker to detect atherosclerosis in the whole circulatory system and has a good prognostic value predicting cardiovascular events [78]. Resnik et al. and O'Hare presented that decreased ABI is associated with an increase in cardiovascular and all-cause mortality, the

accepted value is ABI≤0.9 as high risk for cardiovascular events [79]. A low ABI (≤0.9) indicates the presence of LEAD and commonly refers to atherosclerosis, moreover it was associated with approximately twice higher 10-year CV mortality and major coronary event rate, compared with the overall rate in each Framingham category [80]. Furthermore, asymptomatic LEAD detected by hand-held Doppler has prospectively been found to be associated in men with an incidence of CV morbidity and fatal events approaching 20% in 10 years [6]. The Rotterdam Study found high prevalence of LEAD (19.1%) but rather low prevalence of intermittent claudication (1.6%). The prevalence of LEAD increased dramatically with age from less than 10% in patients between 55 and 59 years of age to nearly 60% in patients being 85 years old or older [81]. In the San Diego Study group, large-vessel LEAD also increased dramatically with age [80]. The population-based Érv Registry was performed in Hungary in 2012, the prevalence of LEAD was 14% among hypertensive patients [82]. Dioszegi et al. found high prevalence of macrovascular complication (48.64%) in diabetic population, 27% of the patients had PAD proved by ABI, in these cases veno-arterial reflex deteriorated significantly [83]. Current cross-sectional and follow-up studies have reported a correlation between the development of diabetic retinopathy and an abnormal ABI [84, 85].

In our study ABI was normal in the young healthy volunteers; only a small portion of the age-matched non-diabetic population had abnormal ABI value, and no one had intermittent claudication. The lower prevalence of LEAD in this group could be due to better controlled hypertension and active lifestyle. More than half of the diabetic patients had mild, moderate or severe peripheral artery disease in our cohort. Codjo et al. found lower prevalence of peripheral artery disease among diabetics in their study [69]. The high prevalence may arise from the fact that retinopathy has been observed in our diabetic group, referring to already existing vascular damages in other vascular beds. Diabetic retinopathy (DRP) is prevalent in patients with diabetic foot ulcer (DFU). Hwang et al. investigated the prevalence of DRP in patients with DFU. In their study about half of DFU patients had proliferative DRP, underlying the necessity of regular checking of retinal examinations [86].

The National Health and Nutrition Examination Survey (NHANES II) determined pulse amplitudes in adults and diminished or absent pulsation of the dorsal pedal artery was found in every fifth case with DM aged 55–74 years [87]. Potier et al. showed that the sensitivity of the standard threshold of 0.9 appears to be lower in diabetic patients, because if there is a peripheral neuropathy or arterial calcification, the efficiency of ABI seems to be limited. In these cases,

other methods should be applied, toe pressure or transcutaneous oxygen pressure measurement, in particular [6, 88].

Beyond the examination of macrocirculation, measuring $tcpO_2$ could provide information on the microcirculation and tissue ischemia. In TASC II tissue hypoxia was defined as $tcpO_2$ <40 mmHg in patients without vascular disease, while patients with critical limb ischemia will almost have <30 mmHg but usually less than 20 mmHg [2, 66, 77, 89]. The WIFI classification in the current ESC/ESVS guideline gives a useful prognostic score assessing limb ischemia based on ABI, absolute ankle pressure and toe pressure or/and $tcpO_2$ value [6]. Faglia et al. identified a cutoff value when revascularization is necessary (34-40 mmHg), while $tcpO_2$ reference data were established for the comparative evaluation of problem wounds by Dooley et al. [67, 90].

Karanfilian et al. demonstrated slightly reduced $tcpO_2$ values in diabetic patients, while Breuer et al. proved that reduction is more impressive when the patient has LEAD besides diabetes [71, 91, 92]. We experienced significantly lower values in the diabetic population at rest and during provocation (both at elevation and stasis) compared to the young volunteers. Although mean values of the diabetic and the age-matched non-diabetic groups were not significantly different, a shift toward worse values could be observed in the diabetic population. Our findings are in line with other studies showing that measurement of $tcpO_2$ gives more information on the microcirculation than the measurement of peripheral blood pressure and provides good discrimination between PAD with (silent) ischemia and non-ischemic status [67, 93]. Based on $tcpO_2$ examination, 15% of the patients suffered from severe limb ischemia without claudication, whose pathology had not been observed before. From those who had ABI >0.9 several people (14.6% of the diabetics) were screened as having limb ischemia based on the low (<30 mmHg) $tcpO_2$ value. There is a reason to use $tcpO_2$ in diabetic population because media sclerosis in the calf arteries can cause falsely higher ABI than it would be if intraluminal pressures were measured. Reduced $tcpO_2$ has been shown in other studies among diabetic patients and the deterioration was more expressed in those cases when patients suffer from diabetic complications, e.g. retinopathy or PAD [91]. Kalani et al. estimated that $tcpO_2$ is a better marker for ulcer healing in diabetic patients with chronic venous ulcer than toe blood pressure, while others revealed that $tcpO_2$ should be a potential predictor of major adverse cardiovascular events among patients with uncomplicated diabetes, and it has a higher predictive value than ABI has [68, 93]. Therefore, detection of limb ischemia by transcutaneous

oximetry should have a greater place in the vascular workup.

To test physical capacity 6MWT was performed, which is a well-known but infrequently used diagnostic procedure in cardiology and angiology. The guidelines approved by the American Thoracic Society in 2002 recognize the six-minute walk test as a useful and safe tool for the evaluation of physical efficiency in individuals with at least moderate chronic obstructive pulmonary disease, heart failure and intermittent claudication [73]. In our study diabetic patients had the lowest walking distance. Walk test can reveal the otherwise hidden limitation of walking ability; it can be an objective measure against patients' subjective estimation of walking distance. $TcpO_2$ measured in vertical leg position of the diabetic patients correlated to the covered walking distance, what may imply that ischemia at rest could predict functional capacity.

Polyneuropathy is a complication of diabetes, which is responsible for more than half of all limb amputations, and it has high economic and quality-of-life costs. Neuropathy has a long asymptomatic latency period; therefore, screening should be crucial. It may mask intermittent claudication in diabetic patients. All patients should be assessed for diabetic peripheral neuropathy starting from diagnosis of T2DM and at least annually thereafter [8]. In the diabetic population significantly decreased tuning fork value could be observed at each localization compared to the other groups. Tuning fork value measured on the first toe was significantly different between young volunteers and non-diabetic elderly patients, what could be due to age-related changes. In a clinical research Lauria et al. found similar results, that is to say: ability to vibration sensing decreases with age due to the decrease of epidermal innervation [94]. Higher values were measured in upper extremities in every group compared to the lower limbs, the difference could be due to the axons being longer in the lower extremities and they are also more susceptible to metabolic and hypoxic damages [95]. Approximately 40% of the diabetic patients showed lower than 4 values in the tuning fork test referring to severe neuropathy, while in the control groups low value could not be detected. The background of the high prevalence of asymptomatic LEAD in our study could be due to polyneuropathy.

Beyond hemodynamic changes, hemorheological alterations play a role in the disturbances of the microcirculation, particularly when the vasodilation capacity is exhausted. Erythrocyte aggregation was examined in diabetic populations in several clinical studies, which demonstrated that RBCs had an increased susceptibility to aggregate. RBC aggregation could

lead to capillary disturbances due to sludge formation [27]. In a previous study higher aggregation was observed among patients with retinopathy [74].

In this study diabetic patients had higher aggregation index and faster aggregate formation compared to the young controls, what is in accordance with previous results [96]. Between diabetic patients and age-matched control group significant difference could not be observed in these parameters, its reason is still unknown, some changes could depend more on age than disease. Disaggregation shear rate (gamma) was significantly higher in the diabetic patients compared to the two other groups.

We could demonstrate the correlation of the 6MWT results and red blood cell aggregation variables. In a recent study Simmonds et al. proved that regular walking improves plasma protein concentrations promoting blood hyperviscosity in DM [97]. It is known that increased RBC aggregation can interfere with oxygen delivery. Dupuy-Fons et al. suggest that hemorheological factors may influence oxygen transfer to distal tissues by maldistribution of blood flow and may have prognostic significance in LEAD [98].

Numerous clinical investigations have shown that erythrocyte deformability improves blood flow in the microvessels and in the large arteries at higher shear rates. Elongation is determined by cell shape and geometry as well as internal viscosity of the RBC. Hyperglycemia results in erythrocyte membrane and cytoplasmic damages, reducing deformability [39]. Good deformability could be a cornerstone of the optimal circulation in the narrow vessels. Reduced erythrocyte deformability has been suggested to play a role in the impaired tissue perfusion in complicated diabetes. Our findings are compatible with the results of other studies that RBC deformability was significantly lower among patients with diabetes compared to subjects without diabetes, and lower RBC deformability might reduce walking capacity through microcirculatory disorders [98-100].

Study limitations

Our study was cross-sectional. In the diabetic population co-morbidities were also observed which could modify macro- or microcirculatory and hemorheological results. $TcpO_2$ was not measured during or after the walk test; and toe pressure was not measured due to unavailability at the beginning of this study.

Conclusion

Lower limb ischemia and microcirculatory disorders are common cardiovascular complications in diabetes mellitus, therefore early diagnosis would be essential to identify patients at higher risk for major adverse cardiovascular events. In diabetes the distal arterial segments are involved and complaints may be missing due to polyneuropathy. LEAD sufferers with diabetes thus may present later with more severe diseases and have a greater risk of limb loss. Our study indicates that the vascular screening of the diabetic population is imperative, physical and instrumental examinations of the lower extremities should become part of the everyday routine. With screening, more asymptomatic organ damages would be recognized in time; moreover, a part of critical limb ischemia and limb loss due to vascular complications of diabetes might be prevented. The measurement of $tcpO_2$ can be a beneficial examination in asymptomatic diabetic patients for detecting microcirculatory disorders and tissue ischemia when ABI is expected to give a false negative result.

The authors declare that they do not have any financial relations either with the manufacturers or distributors of the products in the present studies or with any companies trading rivalling products.

Summary of our scientific results

In vitro hemorheological effects of parenteral agents used in peripheral arterial diseases

1. An *in vitro* model was used to test drugs frequently considered as vasoactive agents in parenteral administration.

2. In this system most of the tested drugs were hemorheologically neutral, although some of them (sulodexide, alprostadil) had slight, but significant positive effect on micro-hemorheological parameters, although this effect in our *in vivo* pilot study was not confirmed. The other investigated drugs had no beneficial effects on the rheological parameters *in vitro*.

3. From this study and previous results found in the literature we may suggest that some positive effects of "vasoactive" infusions *in vivo* could be attributed more to the hemodilution caused by the volume influx than the agent itself. A placebo effect could also be considered.

Lower limb ischemia and micro-rheological alterations in patients with diabetic retinopathy

1. This was the first study that examined lower limb ischemia in patients with diabetic retinopathy by transcutaneous partial tissue oxygen tension as a routine.

2. Patients with diabetic retinopathy have high prevalence of lower extremity arterial disease, which was hidden in a large part of this study population.

3. Absolute ankle pressures and ankle-brachial index values in diabetes could be at any part of the scale from the very low to the higher than normal; the > 0.9 ABI values can mask a severe disease.

4. Tissue partial oxygen pressure measurement has the ability to reveal a population without abnormal ABI but with severe limb ischemia.

5. 6-minute walk test is an easy and cheap method to measure walking capacity in an objective manner.

6. Micro-hemorheological alterations could affect walking distance through disturbing the microcirculation.

7. Diabetic patients would need a multi-vessel/multi-organ approach.

8. Regular screening of lower extremity artery disease and polyneuropathy is encouraged and should be performed routinely in diabetes mellitus.

Acknowledgement

These studies were performed at the 1st Department of Medicine, University of Pecs, Medical School, Pecs, Hungary. I would like to express my appreciation to my program leader, Professor Kalman Toth, for the provided support throughout my work.

I am also very grateful to my project leader, Dr. Gabor Kesmarky, for his continuous help and ideas. Without his valuable advice I would not have been able to accomplish what I have achieved.

I would like to express my sincere gratitude to Dr. Judit Vekasi for her kind assistance and the management of diabetic patients.

I would like to express my special thanks to the former and present Ph.D. students, Dr. Barbara Sandor and Dr. David Kovacs for their scientific and statistical information, and to Dr. Beata Csiszar, Dr. Miklos Rabai, Dr. Kinga Totsimon, Dr. Andras Toth for their great support. I am also thankful to all my colleagues from the 1st Department of Medicine for their helpful advice.

At last, I am thankful to our laboratory assistant, Tapasztone Kornelia Fazekas for her kind help throughout the measurements. My special thank goes to my whole family for their love and encouraging support.

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