

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

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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. At least 50% of all PAD patients are asymptomatic. 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. The main risk factors include smoking, diabetes mellitus (DM), hypertension, age. 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 critical limb ischemia is 10 to 20 times higher in patients with DM, moreover they have a 5-fold higher risk of amputation. The Framingham Study and other epidemiological investigations, have reported that besides conventional cardiovascular risk factors, hemorheological parameters are primary and independent cardiovascular risk factors e.g. hematocrit, fibrinogen and viscosity. Several clinical studies have described an association between hemorheological parameters and macro- or microangiopathies in diabetes. These hemorheological alterations may have a remarkable effect on the whole vascular system causing development of wide range of cardio-, cerebrovascular and peripheral arterial diseases. The alterations of hemorheological parameters in diabetes mellitus and peripheral arterial disease have been described by several studies, 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.

Methods

Hemorheological measurements

Hematocrit (Hct) was measured by microhematocrit centrifuge (Haemofuge Heraeus Instr., Germany). Measurements were performed at room temperature. Plasma viscosity (PV) and whole blood viscosity (WBV) were measured by Hevimet 40 capillary viscometer (Hemorex Ltd., Budapest, Hungary). Red blood cell (RBC) aggregation was measured by Myrenne (MA-1 Aggregometer, Myrenne GmbH, Roetgen, Germany) and LORCA (Laser-assisted Optical Rotational Cell Analyzer; R&R Mechatronics, Hoorn, Netherlands) aggregometers. The blood samples are characterized by the aggregation index (AI), half time ($t_{1/2}$), and threshold shear rate (γ). The temperature was kept at 37 °C. LORCA ektacytometer was used for measuring

erythrocyte deformability. RBCs were deformed by 9 different shear stresses from 30 Pa to 0.3 Pa. RBC deformability is characterized by the elongation index (EI). Deformability results were analyzed by the Lineweaver-Burke nonlinear equation calculating the maximal elongation index (EI_{max}) at infinite shear, and the shear stress ($SS_{1/2}$) required for the half of this elongation.

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. 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

The primary goal was to screen the prevalence of lower extremity artery disease in diabetic patients who were regularly checked for retinopathy. 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

Nowadays several vasoactive drugs are available in PAD treatment, but most of these agents are lacking evidence on the improvement of morbidity and mortality in PAD. 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, 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.

Subjects and Methods

Blood samples collected from 19 non-smoker healthy male volunteers (mean age 27.2 ± 4.3 years) were used in the study. Blood samples were collected from an antecubital vein after a 12- hour fast, hemorheological parameters were determined. The following drugs were investigated in our study: iloprost, alprostadil, pentoxifylline, sulodexide, pentosan polysulphate sodium. 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

Statistical analysis

Data are shown as means \pm 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

Iloprost and alprostadil 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 by iloprost. Alprostadil caused statistically significant increase in the elongation index values at 0.53 and 0.95 Pa shear stresses. 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. Sulodexide caused significantly lower whole blood viscosity, and significantly higher hematocrit/WBV ratio. EI_{max} was significantly higher in sulodexide samples. Incubation with pentosan polysulfate sodium resulted in significantly higher whole blood viscosity and significantly lower Hct/WBV blood viscosity ratio ($p < 0.05$); moreover, we found significantly higher aggregation parameters. Elongation indices did not show any significant changes (Table 1, 2).

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

| Parameter | Control | Iloprost | Alprostadil | Pentoxifylline | 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 | Pentoxifylline | 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. 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.

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. Iloprost added to standard therapy significantly reduced whole blood viscosity and increased physical performance in patients with PAD. 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. Our *in vitro* research showed a slightly but significantly improved RBC deformability at low and medium shear stresses which may improve microcirculation.

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. 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 and reduce RBC aggregation. We could not find any significant effects of pentoxifylline on plasma and whole blood viscosity and RBC aggregation; furthermore, a slightly but significantly decreased deformability could be detected.

According to the literature sulodexide helps protect or restore the integrity and permeability of endothelium against chemical, toxic or metabolic injury; inhibits aggregation and adhesion of platelets, reduces plasma fibrinogen concentrations, reduces plasminogen activator inhibitor-1 and increases tissue plasminogen activator as well as systemic fibrinolytic and thrombolytic activity. It 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. 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.

Pentosan polysulfate sodium is a low molecular weight semi-synthetic heparin-like glycosaminoglycan. 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.

Conclusion

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.

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. The association between diabetes mellitus and PAD has also been well established in several studies. PAD is two to four fold more frequent in diabetes mellitus and it starts 10 years earlier than in non-diabetic subjects. In diabetes PAD is often asymptomatic, therefore systematic screening should be performed. 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. Other non-invasive vascular tests, e.g. toe pressure, tcpO₂ 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. 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.

Methods

105 patients with type 2 DM (mean age 64.64±9.01, 48 females and 56 males), 42 non-smoking young individuals (mean age 25.52 ±3.32 years, 20 females and 22 males) and 35 age-matched non-diabetic volunteers (mean age 61.65±7.6 years, 21 females and 14 males) were enrolled in the study.

Non-invasive arterial diagnostic procedures

Hand-held Doppler, ankle/brachial index

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.

Transcutaneous tissue oxygen pressure

The transcutaneous oxygen pressure was measured with a two-channel oximeter (Tina TCM 4000, Radiometer, Copenhagen, Denmark). Functional and provocational tests (elevation and hanging of the leg) were carried out.

Calibrated tuning fork test

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.

6-minute walk test

In a 6-minute walk test (6MWT) the patient can walk at his own maximal speed on a 30 m long corridor. Maximal walking distance were measured.

Blood sampling, sample preparation

Blood samples were collected for micro-hemorheological measurements (RBC aggregation and deformability).

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 \pm 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.

Results

Non-invasive arterial diagnostic procedures

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 3).

Table 3. 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 | - | - |

In the diabetic population, significantly lower tcpO₂ 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 tcpO₂ on the foot than the young population (Table 4).

Table 4. Results of tcpO₂ 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).

20% of the diabetic patients had normal values and almost other 20% had severe limb ischemia (Table 5).

Table 5. Distribution of the study population in the various tcpO₂ 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 6).

Table 6. 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).

Hemorheological alterations

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 7).

Table 7. 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 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). 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 8).

Table 8. 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; [†]p<0.05, [‡]p<0.0001) (Figure 1 a).

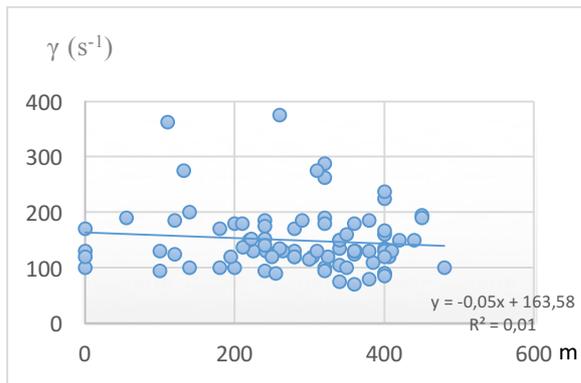


Figure 1. a. Correlation between the maximal walking distance and erythrocyte aggregation characterized by disaggregation shear rate threshold (γ).

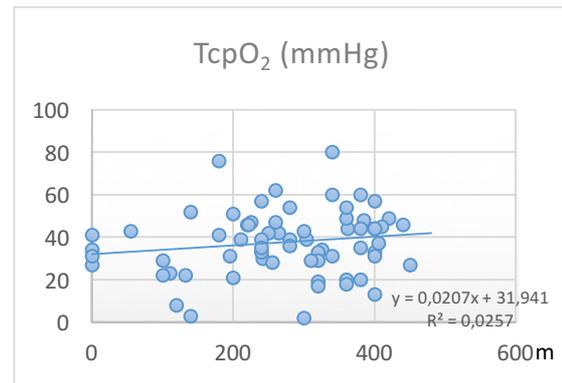


Figure 1. b. Correlation between the maximal walking distance and tcpO₂ values at the stasis on the leg.

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 tcpO₂ 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 1b).

Discussion

The prevalence of lower extremity artery disease is high but largely underestimated. Vascular abnormalities and hemorheological disturbances in diabetes impair the microcirculation provoking organic damages. PAD is an independent predictor for cardiovascular (CV) ischemic events not only in symptomatic but also in asymptomatic diseases. In our study 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. Numerous non-invasive and invasive tests have been designed for diagnosing LEAD. Hand-held Doppler examination is a simple and cheap method to measure blood pressure of the lower extremities. Several researches presented that decreased ABI is associated with an increase in cardiovascular and all-cause mortality.

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. 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. Other researchers found lower prevalence of peripheral artery disease among diabetics in their study. The high prevalence may arise from the fact that retinopathy has been observed in our diabetic group. Measuring tcpO₂ could provide information on the microcirculation and tissue ischemia. We experienced significantly lower values in the diabetic population at rest and during provocation 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 tcpO₂ gives more information on the microcirculation in diabetic population because media sclerosis in the calf arteries can cause falsely higher ABI.

Based on tcpO₂ 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) tcpO₂ value. Six-minute walk test served as a useful and safe tool for the evaluation of physical capacity: in our study diabetic patients had the lowest walking distance. TcpO₂ 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. It may mask intermittent claudication in diabetic patients. In the diabetic population significantly decreased tuning fork value could be observed at each localization compared to the other groups; moreover, significant difference could be observed between young volunteers and non-diabetic patients, what could be due to age-related changes. A clinical research found similar results, that is to say: ability to vibration sensing decreases with age due to the decrease of epidermal innervation. 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. In a previous study higher aggregation was observed among patients with retinopathy. 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. 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 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.

Numerous clinical investigations have shown that erythrocyte deformability influences blood flow at higher shear rates. 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.

Conclusion

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.

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.

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