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Spectral analysis of the blood flow in the foot microvascular bed during thermal testing in patients with diabetes mellitus

Irina Mizeva[a,*], Elena Zharkikh[b], Viktor Dremin[b], Evgeny Zherebtsov[c], Irina Makovik[b], Elena Potapova[b], Andrey Dunaev[b]

[a] Institute of Continuous Media Mechanics, Korolyov 1, Perm 614013, Russia
[b] Orel State University, 95 Komsomolskaya St, Orel 302026, Russia
[c] Aston Institute of Photonic Technologies, Aston University, Aston Triangle, B4 7ET Birmingham, UK

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ABSTRACT

Timely diagnostics of microcirculatory system abnormalities, which are the most severe diabetic complications, is one of the major problems facing modern health care. Functional abnormalities manifest themselves earlier than the structural ones, and therefore their assessment is the issue of primary importance. In this study Laser Doppler flowmetry, a noninvasive technique for the cutaneous blood flow monitoring, was utilized together with local temperature tests and wavelet analysis. The study of the blood flow in the microvascular bed of toes was carried out in the control group of 40 healthy subjects and in two groups of 17 type 1 and 23 type 2 diabetic patients.

The local temperature tests demonstrated that the diabetic patients have impaired vasodilation in response to local heating. The tendency for impaired low frequency pulsations of the blood flow associated with endothelial and neurogenic activities in both diabetes groups was observed. Local thermal tests induced variations in perfusion and its spectral characteristics, which were different in the groups under study. In our opinion, the obtained preliminary results can be a basis for further research and provide a deeper understanding of pathological processes that drive microvascular abnormalities caused by diabetes mellitus.

1. Introduction

In recent years, diagnosis, care and treatment of patients with diabetes mellitus (DM) have been the highest healthcare priorities. In 2017, over 425 million people worldwide were diagnosed with diabetes (estimates from the International Diabetes Federation.) (IDF, 2017). This number is expected to increase to 629 million people by 2045. Clinical observations demonstrate that persistently high blood sugar can damage blood vessels and nerves and that microvascular abnormalities may appear already in the preclinical phases of diabetes (Caballero et al., 1999; Smirnova et al., 2013).

Microcirculation disorders manifest themselves in all parts of the body and affect the functioning of various organs, including kidneys, eyes, cardiovascular system and skin. This significantly reduces the life quality of patients and may lead to their full disability.

Diabetic foot ulcer is a major DM complication, including permanent disability and even amputations at a late stage (Fuchs et al., 2017). Timely diagnosis, monitoring and treatment of the complications reduce the severity of their manifestation and potentially prevent their further development (Schramm et al., 2006).

Assessment of the microcirculation may conveniently be performed in the skin because of its ease accessibility. The cutaneous blood flow can be evaluated using various optical diagnostic methods (Daly and Leahy, 2013), of which Laser speckle, videocapillaroscopy, optical coherence tomography, and laser Doppler flowmetry (LDF) are most frequently used.

LDF (Stern, 1985) allows one to estimate the blood flow in the microvasculature *in-vivo*. It is based on optical non-invasive sensing of tissue using laser light and further analysis of the scattered radiation partially reflected by the moving red blood cells. A great advantage of the LDF technique is its ability to measure blood flow in a local area of tissue with an excellent temporal resolution (Johnson et al., 2014).

Spectral analysis of the LDF signal is widely used to assess the properties of the microcirculation system (Stefanovska et al., 1999). Previous studies have revealed the presence of rhythmic variations in the signal measured by LDF. The energy of individual oscillating components characterizes vascular regulatory mechanisms. By tracing the characteristic frequencies in time the frequency range 0.01–2 Hz was

* Corresponding author.
E-mail address: mizeva@icmm.ru (I. Mizeva).

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divided into five intervals. Later, using longer records, lower frequency components (around 0.007 Hz) were found (Kvandal et al., 2006). The position of frequencies slightly varies from subject to subject, and physical activity and some diseases have weak effects on the boundaries of frequency bands. The pulse (0.45–1.6 Hz) and respiratory (0.2–0.45 Hz) bands carry information about the influence of heart rate and movement of the thorax on the peripheral blood flow. The myogenic mechanism of vascular tone regulation mirrors the response of vascular smooth muscle cells to the transmural pressure. Blood flow oscillations at frequencies (0.05–0.15 Hz) characterize its activity. The neurogenic sympathetic vasomotor activity causes the vessel walls to move with frequency 0.02–0.05 Hz. Slow blood flow waves (0.005–0.0095 Hz and 0.0095–0.02 Hz) reflect the vascular tone regulation due to the endothelium activity, both NO-dependent and independent. These mechanisms were reviewed in detail (Kvernmo et al., 1999; Lancaster et al., 2015).

Regional differences in the cutaneous microvascular function should be taken into account when analyzing variations in skin blood flow (Hodges and Pozzi, 2014; Sorelli et al., 2017). The microcirculatory dysfunction in diabetes usually manifests itself in the feet, and that is why we have examined them in our work.

Functional microcirculation abnormalities appear earlier than the structural ones (Beer et al., 2008). Although many previous studies have shown that the basal cutaneous blood flow is generally lower in patients with diabetes (Walther et al., 2015; Urbancic-Rovan et al., 2004), this fact is attributed to physiological variations in skin blood flow and limitations of the LDF technique (Fredriksson et al., 2007; Mizeva et al., 2016; Zherebtsov et al., 2016). A promising method for the microvasculature functional state monitoring is based on the estimation of dynamic variations in cutaneous blood flow (Geyer et al., 2004; Humeau et al., 2004). The functioning of the microvasculature is often evaluated by analyzing the impact of stress tests: thermal, mental, pharmacological, orthostatic, breath and occlusive. Being noninvasive and easy to implement, thermal tests are most widespread (Dremin et al., 2016; Roberts et al., 2017). Diabetes primarily damages unmyelinated nociceptive C-fibers, which are activated by heating above 42 ° C (Campero et al., 2009). Therefore, + / − heating tests are implemented to assess microvascular abnormalities in subjects with diabetes (Jan et al., 2013; Parshakov et al., 2017).

Both reflexes, vasodilation and vasoconstriction, mirror the function of blood flow regulative mechanisms (Sheppard et al., 2011). Being managed by sympathetic vasoconstrictor nerves (Pergola et al., 1993), the microvascular system is able to decrease the blood flow at low temperature. On the other hand, change in the responsiveness of smooth vascular muscles to sympathetic stimulation during local cooling (Stephens et al., 2001) causes vasoconstriction, which is impaired in patients with DM (Sivitz et al., 2007).

Slow heating (0.5 °C per 5 min or more) (Hodges et al., 2009) reduces the effect of the microcirculatory system response, while at fast heating (0.5 °C per 5 s or less) up to the temperature above 39°C the higher reproducibility takes place (Roberts et al., 2017). In this work, local thermal tests at different temperature (25 °C, 35 °C and 42 °C) were performed consequently. Such sequence of local thermal stimuli promotes pronounced activation of the local regulatory mechanisms of blood flow. In particular, fast local heating up to 35 °C should induce an axon-reflex (Johnson et al., 2014) due to the activation of sensory peptidergic nerve fibers (Stephens et al., 2001). Further heating up to 42 °C provokes the development of vasodilation associated with the release of nitric oxide (NO) from the vascular endothelium (Minson et al., 2001). Stepwise heating up to 35 °C and 45 °C was also used in Vinik et al. (2001); correlations between the blood flow at 35 °C and LDL cholesterol, triglycerides and C-peptide were found. These biomarkers are specific for the metabolic syndrome and, possibly, play a role in the pathogenesis and complications of diabetes.

The aim of this study is to analyze the effect of variations in the main microhaemodynamic parameters on the feet of patients with diabetes type 1 (DM1) and diabetes type 2 (DM2) subject to different modes of heating.

Table 1
The main characteristics of the groups under study.

	Controls n = 40	DM1 n = 17	DM2 n = 23
Sex (M/F)	26/14	10/7	10/13
Foot temperature, °C	27 ± 1	30 ± 1	30 ± 2
Age, years	39 ± 9	35 ± 9	50 ± 6
Diabetes duration, years	–	14 ± 10	7 ± 6
Body mass index, kg/m^2	23 ± 3	25 ± 5	35 ± 5
Fasting glucose, mmol/l		8.1 ± 4.7	9.2 ± 3.5
HbA1c, %		7.9 ± 0.8	8.8 ± 0.9
Total cholesterol, mmol/l		4.6 ± 0.9	5.4 ± 0.9
Creatinine, μmol/l		88 ± 37	74 ± 16
Urea, mmol/l		6.1 ± 3.4	5.7 ± 1.8
ALT, IU/L		27 ± 14	36 ± 18
AST, IU/L		26 ± 10	27 ± 10
Systolic BP, mm Hg	125 ± 9	124 ± 16	141 ± 13
Diastolic BP, mm Hg	80 ± 5	78 ± 7	86 ± 6

2. Materials and methods

2.1. Groups of subjects

The study involved 40 patients from the Endocrinology Department of the Orel Regional Clinical Hospital (Russia) with DM1 and DM2. All the patients were divided into groups according to the report of WHO Consultation (Alberti and Zimmet, 1998). The laboratory, clinical and anthropometric characteristics determined for each subject are given in Table 1. Clinical and laboratory parameters were measured during the standard laboratory procedures. Blood pressure measurements were taken after a 5 min rest of the patient in a sitting position. The average age of patients was 43 ± 10 years. The groups included subjects of slightly different age, but close disease duration.

The control group consisted of 40 healthy volunteers (26 males, 14 females) with a mean age of 39 ± 9 years and without diagnosed diseases of the circulatory system, the musculoskeletal system or the connective tissue. The volunteers with exacerbations in diseases of cardiovascular, pulmonary, and neuroendocrine systems, gastrointestinal tract, liver, kidneys, blood, and any other serious chronic diseases, which could influence the microcirculation system, were excluded from the study, as well as the subjects with an alcohol history and medication or drug abuse.

The study protocol was approved by the Ethics Committee at the Orel State University named after I.S. Turgenev. All volunteers signed the informed consent form.

2.2. Sample collection method

The cutaneous blood flow was examined using the laser Doppler flowmeter (SPE “LAZMA” Ltd., Russia). The Doppler channel is built using single mode laser module with a wavelength of 1064 nm. A fiber optical probe was used to deliver laser light and to register the shifted in frequency radiation reflected from the tissue. The probe radiation power at the output of the fiber probe did not exceed 1.5 mW. The geometric parameters of the fiber probe (light emitting fiber 6 μm in diameter, light-collecting fiber 400 μm in diameter, and a probe gap 1.5 mm) gave the receiving numerical aperture 0.22. The LDF probe sampling volume simulation based on the Monte Carlo technique (Dremin et al., 2017) has shown that the diagnostic volume is about 1.8 mm^3. This provides evidence that the probe is sensitive to the variations of blood flow in the papillary dermis and upper blood net plexus and is able to cover the top part of the reticular dermis.

The optical probe was installed into the hole of the Peltier element,

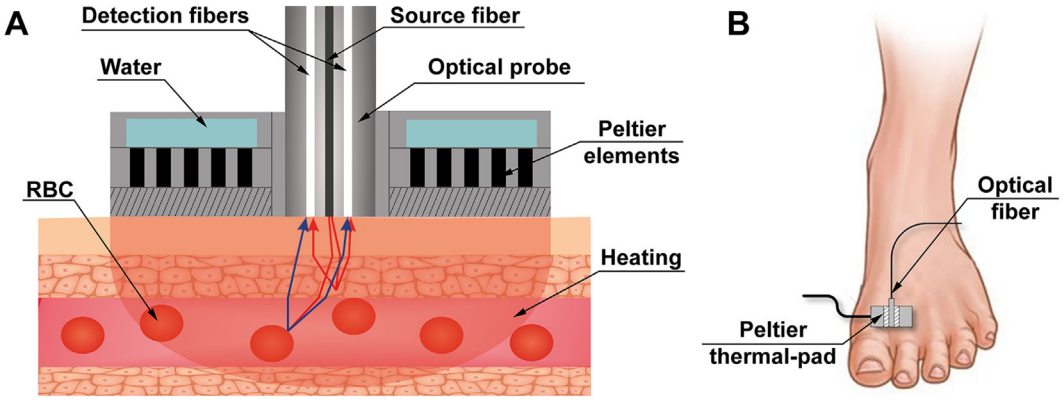

Fig. 1. Coupling of the optical probe with the Peltier element (a); probe location on the human lower limb (b).

and the temperature was controlled by a thermistor with the accuracy of 0.1 °C. This system (the Peltier element and the optical probe) was mounted on the dorsal surface of the foot (Fig. 1a) at a point located on the plateau between the 1st and 2nd metatarsals (Fig. 1b).

Before the measurement taken about 2 h after a meal, the volunteers were adapted for at least 10 min at room temperature. All studies were performed in the subjects lying in the supine position. In the basal state, the subjects had different skin temperatures (Table 1). To unify measurements, we have chosen the cooling temperature of 25 °C. Each study included four steps: the basic test for 4 min, cooling to 25 °C for 4 min, and a few local thermal tests at temperatures of 35 °C and 42 °C for 4 and 10 min, respectively. Thus, the measurement duration for one foot was 22 min. Consequently, both feet were investigated in all patients. The LDF sampling on each leg was collected continuously. In this work, we discuss the results obtained from one leg to exclude long staying in the supine position.

2.2.1. Data preprocessing and analysis

The LDF signal was decomposed using a wavelet transform as:

$$W(\nu, \tau) = \nu \int_{-\infty}^{\infty} f(t)\psi^*(\nu(t-\tau))dt, \tag{1}$$

where * means complex conjugation. The Morlet wavelet written in the form

$$\psi(t) = e^{2\pi i t} e^{-t^2/\sigma} \tag{2}$$

was used for the series expansion in the decay parameter $\sigma = 1$. Integrating the power over time gives the global wavelet spectrum

$$M(\nu) = \frac{1}{T}\int_0^T |W(\nu, t)|^2 dt. \tag{3}$$

We calculated wavelet coefficients for the frequency range 0.01–2 Hz with the logarithmic partitioning on 50 frequency bands. At the first step, we calculated $M(\nu)$ for every record and departed from boundaries and LDF stepwise variations caused by changes of environmental conditions to exclude their influence on the spectrum. The integral wavelet spectra were averaged over the group. For each frequency band, we obtained energy distribution, which was compared in health and pathological groups. The frequency bands corresponding to different physiological mechanism are shown on the plots for the reference.

For reliable statistic one should ideally include 10 cycles for each of the frequency under investigation. We have 4 min recording for each of the first 3 phases. That is why the reliable results can be obtained only for frequencies higher than 0.04 Hz. For lower frequencies, the results are presented to demonstrate the tendency of qualitative data only.

The Mann-Whitney test was used to compare the intergroup results and the Wilcoxon statistical test – to evaluate the intragroup variations. Finally, in order to get robust results, we have performed sample size estimations for minimization of type two error:

$$n = 2SD\frac{(Z_{\alpha/2} + Z_\beta)^2}{d^2} \tag{4}$$

where is the *SD*– standard deviation; $Z_{\alpha/2} = 1.96$ at type 1 error of 5%; $Z_\beta = 0.84$ at 80% power; d is the difference between mean values (Charan and Biswas, 2013).

The data processing procedure was carried out using original Mathematica 8.0, Wolfram Research.

3. Results

An example of the collected LDF samples is presented in Fig. 2. Thermal tests provoke significant variations both in the average perfusion (Fig. 4) and its oscillation component (see the third experimental stage in Fig. 2). The statistical analysis (Fig. 3) demonstrates close values of *P* without any significant differences in the basal state. Cooling

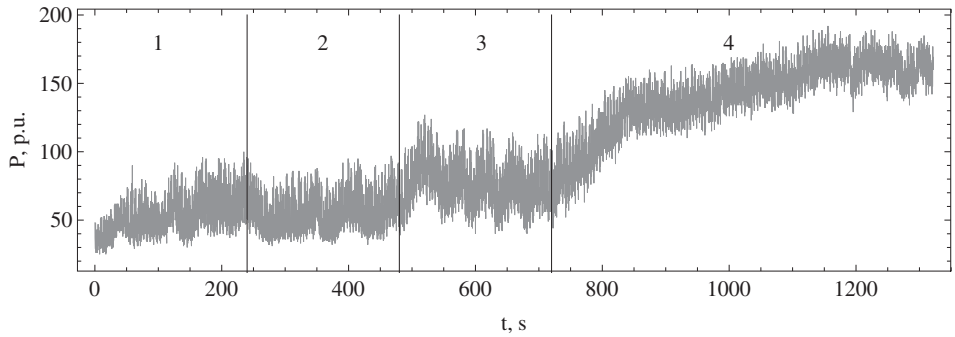

Fig. 2. Typical LDF sample collected from the patients with diabetes (right foot, diabetes duration - 30 years). Numbers indicate experimental stages: 1 - basal conditions, 2 - cooling, 3- first heating up to 35 °C, and 4 - second heating up to 42 °C.

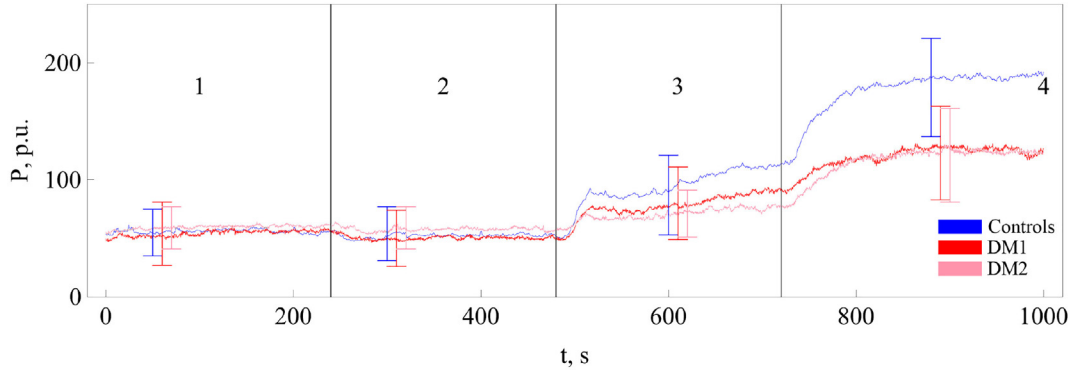

Fig. 3. Dynamics of perfusion averaged over all measurements (blue - controls, red - patients with DM1, light red - patients with DM2). First, we applied the moving average filter with a window of 0.25 s and then estimated a mean value at each instant. Error bars indicate a mean standard deviation at a certain stage of the experiment. Numbers show experimental stages: 1 - basal conditions, 2 - cooling, 3 - first heating up to 35 °C, 4 - second heating up to 42 °C. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

provokes weak vasoconstriction and heating – significant vasodilation. Note that the vasodilation dynamics varies in groups (Fig. 3). All measurements showed a peak at the beginning of heating caused by the axon-reflex. The highest rate of vasodilation is found in the control group and this characteristic is impaired in both diabetic groups. Moreover, the heating clarified the difference between groups, and perfusion of the heated skin significantly differs in the groups of healthy and diabetes subjects, but is similar in two diabetes groups.

3.1. Basal conditions

The lowest perfusion under basal conditions was observed in the control group ($P = 53 \pm 18$ p.u.). The patients with both types of diabetes had slightly higher perfusion, 54 ± 27 p.u. in DM1 and 58 ± 20 p.u. in DM2. Both diabetes groups had the impaired amplitude of perfusion oscillations in the frequency range (0.012–0.045) Hz (Fig. 5) in comparison with the control group. These frequencies fall into the intervals, which correspond to neurogenic and endothelial vascular tone regulation mechanisms. The result is not statistically reliable due to the short data sample. Fluctuations in the range of 0.5 to 1 Hz were weaker in both diabetes groups as well. Moreover, the oscillations of these frequencies were significantly lower in patients with DM2 than in patients with DM1.

3.2. Local cooling

Local cooling-induced vasoconstriction causes the variation in the spectral properties of LDF signals. The averaged perfusion is 46 ± 16 p.u. in the control group, 50 ± 23 p.u. in the DM1 group, and 55 ± 15 p.u. in DM2. To study the phenomenon of spectral variation, we estimated the difference between $M(\nu)$ in basal conditions and at cooling for every subject. The results obtained are presented as a box-whisker plot (Fig. 6). The spectral characteristic of LDF samples of healthy subjects only slightly varies in response to cooling.

At exposure temperature of 25 °C a significant reduction in oscillations was observed in the frequency band of 0.05 to 0.14 Hz in patients with DM1. This frequency band falls within the range associated with the myogenic activity. The spectral energy of the LDF signal of DM2 patients in this frequency band remained unchanged. Observed trend toward increased oscillations of the 0.02–0.04 Hz frequency band associated with the neurogenic activity during cooling in DM2 should be checked on larger samples. Note that the variation in the amplitude of pulsations in these frequency bands caused by local cooling is significantly different in two diabetic groups.

3.3. Local heating (35 °C)

Local heating up to 35 °C provoked vasodilation. The difference of averaged perfusion became significant between the examined groups,

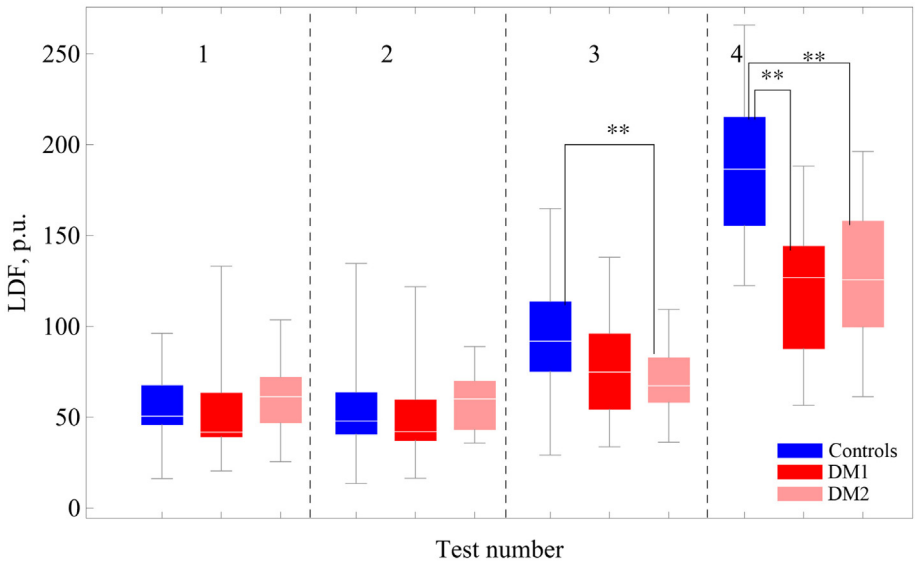

Fig. 4. Box-Whisker diagram of mean perfusion during 4 experimental stages: 1 - basal conditions, 2 - cooling, 3 - first heating up to 35 °C, 4 - second heating up to 42 °C. By stars we mark the level of significance estimated using Mann-Whitney test (** - $p < 0.01$). Increase of perfusion during tests 3 and 4 was also significant. In both groups, tests provoked significant variation of perfusion (the level of significance was estimated using Wilcoxon test, $p < 0.001$).

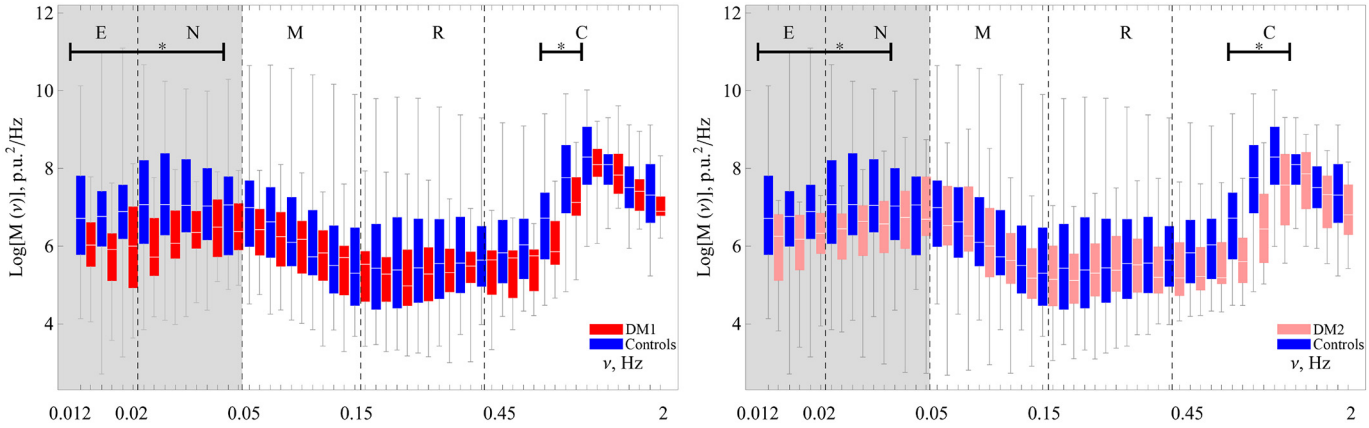

Fig. 5. Averaged spectra of LDF samples in basal conditions. Thick lines in the upper parts of the plot indicate the frequency band, where $M(\nu)$ is significantly different ($p < 0.05$). The low frequency part of the spectra (shaded with gray) has insufficient statistics and is shown just to demonstrate the tendency.

and perfusion increased to 92 ± 28 p.u. in healthy subjects and still remained slightly lower at the level of 79 ± 30 p.u. in DM1 and 67 ± 16 p.u. in DM2.

At this stage, oscillations in the frequency band 0.05–0.45 Hz increased in the control group and in both diabetic groups (Fig. 7). There is a sharp peak in the spectra variation diagram for the controls at the frequency close to 0.14 Hz. The variation mentioned above is significantly lower in both patient groups in comparison with the control group. The smallest reaction was observed in DM1 subjects. Together with oscillations corresponding to the myogenic activity, low frequency oscillations increased in all three groups. The difference in the amplitude of oscillations in basal and heated states was found to be significant for all three groups. Taking into account the relation between pulsatile and averaged components of LDF signal (Mizeva et al., 2016) and rising the average perfusion due to heating, it is difficult to explain this result. On the other hand, averaged perfusion in the groups with diabetes is statistically equal ($p > 0.05$), but it is worth noting here that the amplitude of oscillations in the endothelial frequency band in pathological groups varies weaker. Hence, we can conclude that the mechanisms involved in the low frequency modulation of the cutaneous blood flow are strongly related to DM1.

3.4. Prolongated local heating (42 °C)

The next stage of the experiment was prolongated heating up to 42 °C. Higher temperature provokes stronger vasodilation, so the mean *P* rose up to 190 ± 27 p.u in the control group. Vasodilation response was impaired in both patients groups (128 ± 38 p.u. in DM1 and 122 ± 38 p.u. in DM2). Local heating up to 42 °C caused an increase in all frequency bands (Fig. 8). Note that the amplitude of oscillations is lower in both diabetes groups in comparison with controls, and it is significant for high frequency pulsations of patients with DM2.

To minimize type II error, the necessary number of subjects (sample (4)) was estimated in order to get all parameters suggested as significantly different. We analyzed mean perfusion at various stages of the temperature test, energy of LDF fluctuations in all frequency bands under consideration, and variations in this energy due to differences in temperature. It turned out that the spectra differences in the cardiac frequency band are strictly justified by considering the samples close to 40 subjects in the group. Variations in the myogenic vascular tone regulation mechanisms caused by cooling are justified when testing 55 subjects. The same number of subjects is required for the quantification of myogenic-associated oscillations caused by heating to 35 °C. The perfusion difference due to heating up to 42 °C is statistically meaningful in case of 12 subjects in the sample. Thus, in order to obtain the robust statistical results of spectral characteristics, it is necessary to have at least 55 subjects in a sample.

4. Discussion

Variations in the cutaneous blood flow caused by local cooling and heating were analyzed; measurements were performed using LDF. In

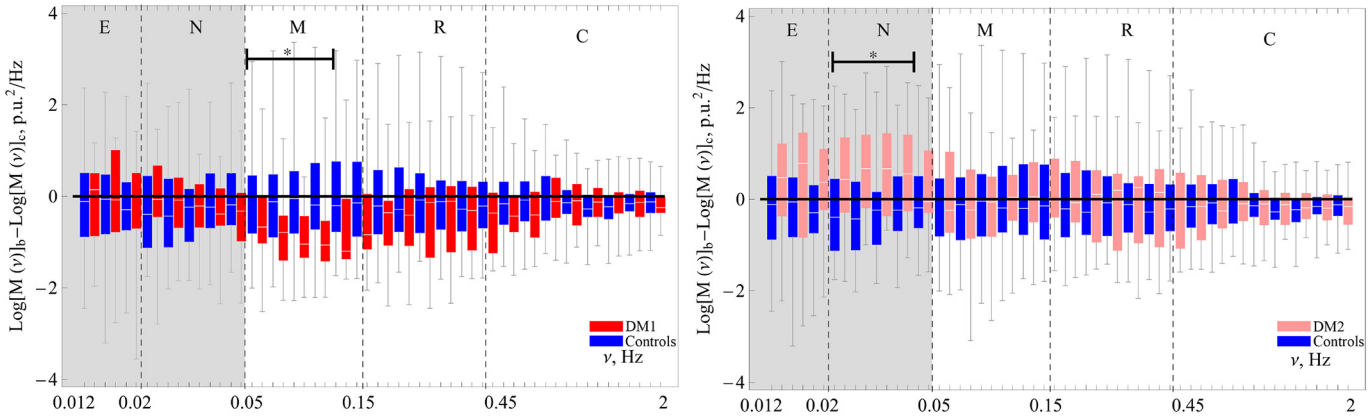

Fig. 6. Variation of the spectral energy caused by cooling. For every frequency, we calculated $M(\nu)_c$ during cooling ($M(\nu)_c$) and in basal conditions ($M(\nu)_b$), then estimated their difference for every frequency $M(\nu)_c - M(\nu)_b$ and for all LDF samples. After that, we constructed the Box-Whisker diagram. The thick lines in the upper part of the plot show the frequency bands, where the variation of energy of pulsation is significant ($p < 0.05$). Low frequency part of the spectra (shaded with gray) is shown just to demonstrate the tendency similar to Fig. 4.

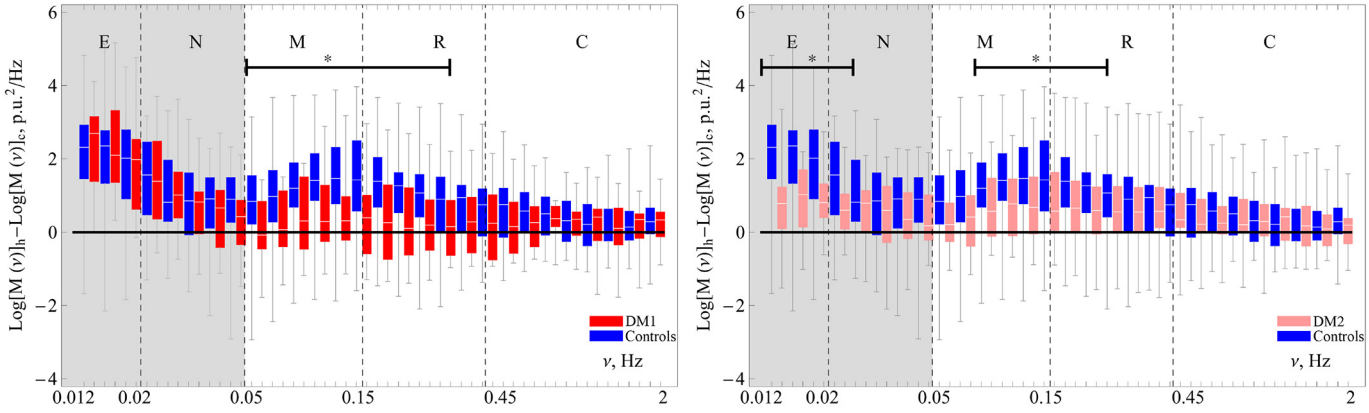

Fig. 7. Variation of the spectral energy ($M(\nu)_h - M(\nu)_b$) caused by heating up to 35 °C. The plot algorithm is similar to the one used in Fig. 6. Low frequency part of the spectra (shaded with gray) is shown just to demonstrate the tendency similar to Fig. 4.

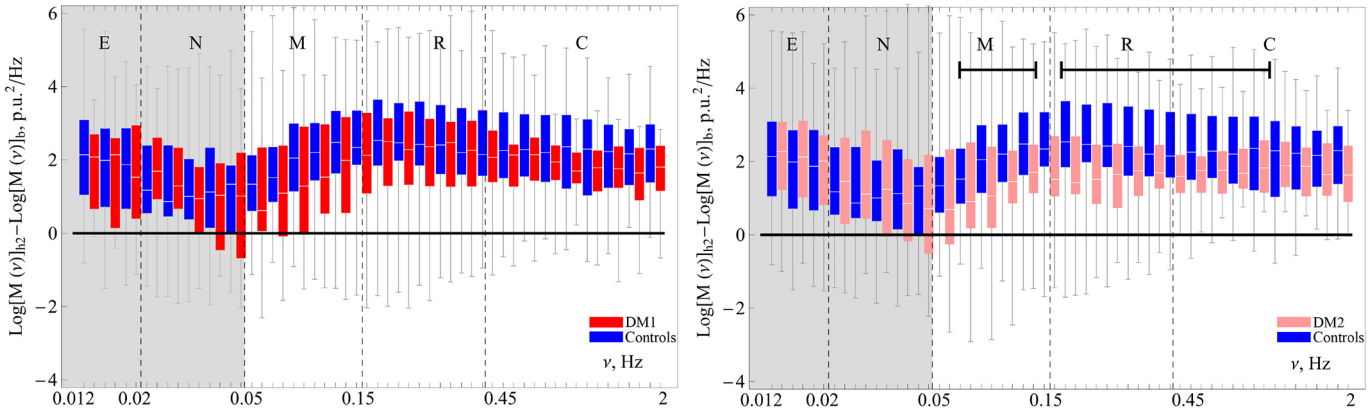

Fig. 8. Variation of the spectral energy ($M(\nu)_h - M(\nu)_b$) caused by heating up to 42 °C. The plot algorithm is similar to the one used in Fig. 6. Low frequency part of the spectra (colored with gray) is shown just to demonstrate the tendency similar to Fig. 4.

basal conditions, all subjects had a similar level of perfusion, which was slightly higher in DM2 patients. These results match previous studies, where the effect of diabetic neuropathy on perfusion was explored (Schramm et al., 2006; Jan et al., 2013).

Even in rest conditions, the analysis of blood flow oscillations revealed a significant difference in the microhaemodynamic parameters of healthy and pathological subjects. The amplitude of 1 Hz oscillations was lower in both DM groups than in the controls in basal conditions. Since the local microvascular tone regulation mechanisms are not involved in modulation of cardiac activity, we assume that this difference is related to the morphological abnormalities of the microvascular system in diabetes. The lowest energy of 1 Hz pulsations was observed in patients with DM2, and in DM1 this energy was slightly higher. However, both values are markedly lower compared to the control group. The cardiac stroke volume (Devereux et al., 2000) in patients with DM is higher than in healthy ones, and therefore one can conclude, that cardiac wave is dumped stronger in DM patients than in healthy ones by the cardiovascular system. Metabolic syndrome, insulin resistance, impaired glucose tolerance and accumulation of advanced glycation end products are positively correlated with increased arterial stiffness (Zieman et al., 2005). Therefore, we suggest that the difference in blood flow oscillations associated with cardiac activity indirectly characterizes the elastic properties of vessels and indicate the increased arterial stiffness (Jaiswal et al., 2013) of diabetic patients. The results obtained are consistent with Lal and Unni (2015) where statistically significant differences were found in the contribution of relative energy in the cardiac frequency band to the total blood flow between the control group and subjects with DM2. The authors advanced a similar assumption that these changes are due to an increase vascular resistance caused by vasoconstriction.

The response of smooth vascular muscles to sympathetic system stimulation during local cooling (Stephens et al., 2001) provokes vasoconstriction. The spectral characteristics of LDF signals in controls are practically not disturbed by cooling; small variations are observed at the left end of the neurogenic frequency band. Patients with DM1 demonstrated a decrease in myogenic oscillations caused by local cooling.

Local mild heating initiates the sequence of reflexes, which leads to vasodilation (Johnson and Kellogg, 2010). At the beginning of heating we observed a local peak on the perfusion-time curve associated with the axon reflex; vasodilation and its rate were impaired in DM patients in comparison with controls. Further, after the local minimum on the perfusion-time curve, one can see a repeated increase in perfusion associated with nitric oxide (NO) release (Johnson and Kellogg, 2010). Perfusion at this stage was impaired in DM patients, as in Stevens et al. (1994, 1995). Having the highest perfusion in basal conditions, the patients with DM1 have the lowest one under local heating conditions. In Mizeva (2017), such a behavior was interpreted as a low reserve of the microcirculation system in pathological conditions. The LDF record during this test is nonstationary and its slow variations are related to the axon-reflex peak in the first part of the test and to endothelial activity in its second part. For this reason as far as signal length is short for the analyses of slow perfusion oscillations, the discussion of the low-frequency part of the spectra is dropped from the consideration. We revealed the increase of myogenic activity in all groups; its highest variation, which was observed in the control group, was accompanied by a sharp peak at a frequency close to 0.14 Hz. Similar behavior observed in Sheppard et al. (2011) was associated with high pre-capillary pressure and the stretching of arterioles, causing myogenic oscillations.

DM patients had the lower response of myogenic activity to heating.

Prolongated heating induces NO-mediated vasodilation, which is lower in DM patients and associated with endothelial dysfunction (Shi and Vanhoutte, 2017). Endothelial dysfunction is characterized by the decreased NO production and bioavailability, the increased production of vasoconstrictors (endothelin-1), the high level of oxidative stress and the process of angiogenesis, which are typical for diabetes. The long term exposure to high temperature causes an increase in perfusion and variations in its spectral properties. The spectra of LDF signals for healthy volunteers vary weaker in the neurogenic frequency band compared to another frequency bands. This is indicative of a key role of endothelial activity in vasodilation at this stage. In both diabetes groups, the vasomotions were impaired in comparison with controls.

To sum up, we have applied a modified version (Mizeva et al., 2017) of the commonly used approach to analyze the spectral properties of LDF signals. The wavelet spectra of LDF signals are frequently undergone the additional post processing procedure such as calculating the average density in the frequency band (Stefanovska et al., 1999). Our approach allowed one to avoid signal post processing, to compare the raw spectra of signals and, finally, to determine frequency bands having significantly different characteristics.

5. Study limitation

It is shown that the majority of our results should be considered as preliminary estimates because the study has some limitations. The issue concerning groups' content is rather difficult and disputable. The key point in the analysis of age, sex and disease duration effects on both micro- and macrohaemodynamic parameters is to determine which factor has the strongest impact on the measured characteristics. We applied Fisher's exact test to the groups, which allowed us to reveal the following association between DM1 and DM2 groups; the outcomes of male and female patients are considered to be statistically insignificant ($p = 0.52$). Close results were obtained for controls and DM1 groups ($p = 0.77$) and controls and DM2 ($p = 0.12$). Another question concerns age and disease duration. As most of DM1 patients are young (35 years old in our study) and DM2 usually develops later (average age in our study is close to 50 years), it is quite difficult to compare these two groups. We compared healthy volunteers with DM1 patients of almost the same age and then compared two diabetic groups with the same duration of diabetes (according to survey), but unavoidable different ages. By analyzing additional data, we obtained the same results in young and aged controls. Hence it can be concluded that we have developed a new rational approach.

The approved protocol of LDF signal registration made the spectral analysis in the experiments stages difficult. This work has sufficient limitation due to the LDF sample length, which leads to inaccurate interpretation on low frequency oscillations. For this reason, we can demonstrate only a tendency for spectral variations. Among these preliminary results, the most interesting finding concerns a different behavior of NO associated vasomotions in DM1 and DM2. Further studies are needed to validate the mechanisms and clinical appropriateness of the vasomotion analyses.

High parameters divergence together with low difference leads to low robustness of the results. Spectral characteristics of LDF samples are characterized by high intra-subject variability. Our estimations have shown that the sample size should be estimated in at least 55 subjects in every group under consideration to minimize type II error. It should be noted that the presented results, despite the low power, still have clinically significant differences. We mention that the number of studied subjects in this research with DM1 and DM2 was relatively small. As to the novelty of approach, the study protocol and the spectral analysis of signals do not rely on any previous experience and sample size estimations.

This study is necessary for planning more expensive and large-scale clinical trials. At this stage of research the hypothesis about the possibility of assessing vasomotion disorders with the help of blood flow spectral analysis during a local heating test has been verified. Within the framework of the work, the strengths and weaknesses of the proposed experimental technique were revealed. The applicability of methods and tools for participants was assessed and specific estimates of the sample size were provided to obtain sufficient statistical power of the study.

Duality of Interest No potential conflicts of interest relevant to this article were reported.

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