ISSN 0030-400X, Optics and Spectroscopy, 2017, Vol. 123, No. 6, pp. 955–964. © Pleiades Publishing, Ltd., 2017.
Original Russian Text © E.V. Potapova, V.V. Dremin, E.A. Zherebtsov, I.N. Makovik, E.V. Zharkikh, A.V. Dunaev, O.V. Pilipenko, V.V. Sidorov, A.I. Krupatkin, 2017, published in Optika i Spektroskopiya, 2017, Vol. 123, No. 6, pp. 946–956.

GEOMETRICAL AND APPLIED OPTICS

A Complex Approach to Noninvasive Estimation of Microcirculatory Tissue Impairments in Feet of Patients with Diabetes Mellitus using Spectroscopy

E. V. Potapova[a,*], V. V. Dremin[a], E. A. Zherebtsov[a,b], I. N. Makovik[a], E. V. Zharkikh[a], A. V. Dunaev[a], O. V. Pilipenko[a], V. V. Sidorov[c], and A. I. Krupatkin[d]

[a] *Orel State University Named after I.S. Turgenev, Orel, 302026 Russia*

[b] *Aston University, Aston Institute of Photonic Technologies, School of Engineering and Applied Sciences, B4 7ET Birmingham, United Kingdom*

[c] *LAZMA Scientific Production Enterprise, Moscow, 123458 Russia*

[d] *Priorov Central Research Institute of Traumatology and Orthopaedics, Moscow, 127299 Russia*

**e-mail: potapova_ev_ogu@mail.ru*

Received June 6, 2017; in final form, July 18, 2017

Abstract—The possibility of a complex approach for studying changes in the system of blood microcirculation and metabolic processes in the biotissue of lower extremities using optical noninvasive methods of laser doppler flowmetry (LDF), fluorescence spectroscopy, and diffuse reflectance spectroscopy in combination with different modes of heating tests has been assessed. Seventy-six patients with type 2 diabetes mellitus, with 14 patients having visible trophic foot impairments, and 48 healthy volunteers have been examined. The parameters of LDF signals and spectra of fluorescence intensity and diffuse reflectance for foot skin have been analyzed. Statistically significant differences in the recorded parameters between the groups under study have been found. It has been concluded that combined application of noninvasive methods of spectroscopy could be used for diagnostics of complications both upon the occurrence of preliminary symptoms of diabetes, when pathological changes are still reversible, and in the presence of impairments to prevent aggravation of the disease and select an adequate correction of the treatment.

DOI: 10.1134/S0030400X1712013X

INTRODUCTION

According to data published by the International Diabetes Federation (IDF), 415 million people in the world suffer from diabetes mellitus and more than 300 million people with impaired glucose tolerance are at high risk. It is predicted that by 2040 there will be 642 million people with diabetes mellitus (DM). Hyperglycemia in DM can lead to serious diseases that affect the hearth and blood vessels, eyes, kidneys, and nerves by causing a few complications, which result in a high risk of disability and are life-threatening.

One serious complication of DM that significantly decreases quality of life is diabetic foot syndrome (DFS). DFS unites a number of foot pathologies due to the damage of nerves and blood vessels. This may cause infections and ulcers, which increases the risk of lower-extremity amputation and leads to early disability and high economic costs for treatment and medical and social rehabilitation. As a rule, surgical treatment is long preceded by trophic disturbances in the form of foot ulcers [1]. Recent studies note that early diagnostics and treatment that include increased patient monitoring reduce the occurrence of complications and, at early preclinical stages, might reverse them.

All of these make early diagnostics of pathologies of lower extremities in DM extremely important. It has been suggested to use methods of spectroscopy, laser doppler flowmetry (LDF), fluorescence spectroscopy (FS), and diffuse reflectance spectroscopy (DRS), which have several advantages: painlessness of the procedures, quick results, an absence of expensive reagents and expendable materials, and minimum effect on the subject being investigated and its properties [2, 3].

One of the causes of angiopathy of the lower extremities in DM (diabetic angiopathy) and, as a result, trophic disturbances in DFS is impaired microcirculation. Clinical studies demonstrate that patients with diabetes mellitus have reduced oxygen supply to lower extremities [4]. Hypoxia syndromes that are associated even with minimum arterial insufficiency lead to irreversible progress of disorders of the tissue metabolism at different times, which is particularly clearly manifested in patients with diabetic foot ulcers.

Though the problems of impaired blood microcirculation in lower extremities in DM are studied in detail, their contribution to DFS is still a subject of study. According to [5], identification of impaired blood microcirculation in a trophic ulcer, as compared to the skin of the same foot region where the ulcer is, is of particular significance. This is necessary for monitoring of the local blood flow during healing of a trophic defect. In addition, in severe cases, determination of the severity of ischemia in a damaged extremity upon diabetic angiopathy plays an important role in the surgical approach for treatment of lower extremities with purulonecrotic lesions [6].

To determine the peripheral blood flow in patients with DM, methods of dopplerometry and dopplerography are most often used and the parameters of the ankle-brachial index (ratio of the systolic pressure in the shin artery to that in the brachial artery), which reflect a decrease in the arterial blood flow, are assessed [7]. Tissue oxygenation is determined by transcutaneous oximetry (TcpO2); however, although this method is considered a gold standard for determination of the oxygen status of tissues, it has not been widely applied in the diagnostics in hospitals due to physiological, methodological, and technical obstacles. At the same time, the informativity of this method and the possibility of using it in the presence of infectious inflammation of foot, peripheral edemas, and other concomitant pathological states that affect the results of the study are still undetermined [4].

Noninvasive optical methods that have been proposed, LDF, FS, and DRS, make it possible to obtain information on the blood filling of blood vessels, concentration of skin chromophores and fluorophores, and intensity of metabolic processes in the skin [2, 3, 8], among other things, for patients with endocrine disorders.

Analysis of tissue oxygenation by DRS is based on the difference in the absorption spectra of the main tissue chromophores, oxyhemoglobin (*HbO*2) and deoxyhemoglobin (*Hb*). By recording a diffuse reflectance spectrum, it is possible to determine the oxygen saturation of the tissue [9], among other things, in diabetic pathologies [10, 11] using the spectrum shape (its slope, area under the curve) on the basis of mathematical simulations of the relation between the reflectance and concentrations of the mentioned chromophores. In addition, DRS, in contrast to transcutaneous oximetry can be applied to study processes that proceed directly in areas of severe metabolic impairments such as trophic ulcers in diabetes [12].

LDF, which is based on tissue probing by laser radiation and analysis of radiation that is reflected and scattered by red blood cells moving in the tissue, makes it possible to assess the perfusion in a microcirculatory part of the blood bed in patients with DM in vivo [13, 14]. Additional possibilities for evaluation of changes that occur in the system of blood microcirculation are provided by the spectral analysis of the recorded signal [15, 16]. Application of this mathematical apparatus makes it possible to analyze different mechanisms of regulation within a wide frequency range (0.0095–1.6 Hz). An increase in the informativity of the diagnostics is promoted by application of different stress tests in combination with LDF. This makes it possible to broaden the diagnostical possibilities of the method and makes it possible to determine not only the general functional state of the microcirculatory bed, but also its spare and adaptive capacities [17].

It is known that changes in the activity of enzymes of the respiratory chain are considered to be one of the consequences of the impairments in cell functions that are observed in diabetes mellitus [18–20]. Many investigations that study the metabolism of epithelial tissues, including skin, using fluorescence microscopy and visualization methods are being carried out today. The most probable changes in different pathologies are changes in the accumulation of NADH (reduced form) and FAD (oxidized) coenzymes. Detection of these changes using fluorescence spectroscopy is among the most promising areas of in vivo diagnostics.

Recent reviews [21, 22] and some other studies demonstrate that changes in the NADH fluorescence have been estimated in individual cells [23, 24], tissue sections [25], and organs [26, 27]. Despite the successful investigations of the NADH and FAD fluorescence that have been carried out in the last 50 years, only a relatively small number of them have been performed at the organ level. Moreover, the results of experiments on a certain organ or part of the organism are more likely to be successfully used in clinical practice than in research at the cellular and subcellular levels.

In recent years, it has been demonstrated that, in DM, in conditions of prolonged hyperglycemia, protein glycation is found to be elevated, which is accompanied by an increase in the so-called "advanced glycation end products" (AGEs) that are involved in the glycation of collagen and other proteins of the capillary membrane and skin [28–31]. Advanced glycation end products (e.g., *N*ε-(carboxymethyl)lysine, *N*ε-(carboxyethyl)lysine, and pyrralin) that possess intrinsic fluorescence include pentosidine, which is responsible for the formation of cross links between collagen fibers [32–34].

Thus, from the point of view of fluorescence analysis, it is reasonable to consider a complex parameter that would describe oxidative metabolic processes (changes in the content of NADH and FAD) and carbon metabolism (accumulation of AGEs) in the tissue.

The proposed combined application of the methods of LDF, FS, and DRS in diagnostics may make it possible to fully analyze pathological changes in the tissues of patients with DM, which would potentially increase the informativity and reliability of the

obtained diagnostic results and make it possible to reveal impairments in the system of blood microcirculation and metabolic processes of the foot biotissue at earlier stages.

This study was aimed at an experimental investigation and analysis of potential possibilities of combined recording of parameters of blood flow, fluorescence, and reflectance spectra of the skin by spectroscopy in order to determine microcirculation and tissue impairments in the lower extremities of patients with diabetes mellitus.

MATERIALS AND METHODS

The experimental studies involved 76 patients (28 men and 48 women) with diabetes mellitus in the Endocrinology Department of the Orel Regional Clinical Hospital (Russia). The study included patients with type 2 DM with a prolonged course of the disease and highly glycated hemoglobin, which were at risk for the development of the diabetic foot syndrome. All the patients were divided into two groups: a group of patients with visible trophic foot pathologies (eight men and six women, average age of 53 ± 13 years) and a group of patients without visible trophic pathologies (20 men and 42 women, average age of 54 ± 10 years). In addition to visible trophic ulcers, the first group of patients had a more prolonged course of diabetes, higher creatinine and urine values (which can indicate kidney dysfunctions), and a higher percentage of diagnosed complications (diabetic polyneuropathy, diabetic retinopathy, diabetic nephropathy, diabetic microangiopathy of lower extremities). The exclusionary criteria for carrying out experimental studies were acute periods of diseases of the cardiovascular and respiratory systems, gastrointestinal tract, liver, kidneys, and blood, diagnosed edemas, varicose veins, and thrombophlebitis, i.e., states that could affect the diagnostic results. The control group included 48 health volunteers (16 women, 32 men) with an average age of 46 ± 6 years.

Experimental studies were carried out according to a developed research protocol that was approved by the Ethics Committee of the Orel State University Named after I.S. Turgenev (November 3, 2015, record of meeting no. 7). All the participants of the experimental studies were acquainted with the content of the study and signed an informed consent form indicating their willingness to participate in the measurements.

In complex experimental studies, the fluorescence of the biotissue (by FS), tissue perfusion by blood (by LDF), and coefficient of the diffuse reflectance of the skin in the spectral range from 500 to 600 nm (by DSR) were assessed. All the studies were carried out in a supine position 2 h after food intake. The test subject adapted to the environment for no less than 10 min. The studies had two stages using two experimental setups without a change in the position of the patient.

At the first stage of the study, the diffuse reflectance spectra of the foot skin of patients were recorded using a setup, the scheme of which is given in Fig. 1. It includes a lighting unit, A broadband tungsten halogen radiation source (HL-2000-HP-232R, Ocean Optics, United States). A fiber-optic probe (R400-7, Ocean Optics, United States) that had seven fibers (six illuminating fibers that are located around one reading fiber) was used to deliver irradiation from the lamp and to collect the reflected signal. The diagnostic volume and probing depth for this probe within the spectral region of the hemoglobin Q bands (540–580 nm) are 1.0–1.3 mm^3 and 0.4–0.5 mm, respectively, as shown in [35]. In addition, the area of the study includes the epidermis and papillary dermis. Reflected light that is collected by the optic fiber is directed to a CCD of a small-size spectrometer (FLAME, Ocean Optics, United States). To control the system, a personal computer was used, with data storage and reproduction being carried out using the Ocean View software (Ocean Optics), which was supplied with the spectrometer.

To record reflectance spectra, an initial calibration was carried out beforehand. The reflectance spectra were calculated according to the formula

$$R(\lambda) = \frac{R_t(\lambda) - R_b(\lambda)}{R_{\text{PTFE}}(\lambda) - R_b(\lambda)},$$

where $R_t(\lambda)$ is the measured diffuse reflectance (DR) in the biological tissue, $R_{\text{PTFE}}(\lambda)$ is the measured DR of the WS-1 diffuse reflectance standard, and $R_b(\lambda)$ is the background spectrum that was obtained with the light source turned off.

This normalization makes it possible to exclude the effect of irradiation intensity of the source and sensitivity of the receiver on the measurements of spectral dependences. The distance from the fiber-optic probe to the studied surface was the same upon the calibration and measurements.

The diffuse reflectance spectra were recorded in the control point of the line between the first and second metatarsal bones of the foot (the schematic arrangement of the fibers is given in Fig. 1). Moreover, patients with visible trophic impairments in the form of ulcers had additional diffuse reflectance spectra recorded directly in the ulcer and at 1 cm from it (the so-called “intact region”).

At the processing stage, hemoglobin index H and degree of its oxygenation Y were calculated and analyzed according to the method that was suggested by J.W. Feather et al. [36, 37] and constructed on the

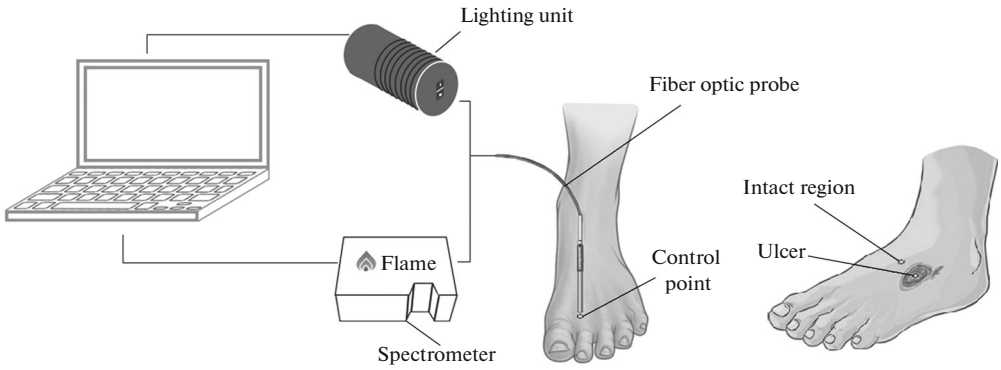

Fig. 1. Scheme of an experimental setup for analysis of the diffuse reflectance spectra of skin.

basis of measurements of the gradient of the spectrum of optical density in the region of 535–575 nm:

$$H = 100\left(\frac{\mathrm{OD}_{544} - \mathrm{OD}_{527.5}}{16.5} - \frac{\mathrm{OD}_{573} - \mathrm{OD}_{544}}{29}\right);$$

$$Y = \frac{5.1\times10^3}{H} \times\left(\frac{\mathrm{OD}_{573} - \mathrm{OD}_{558.5}}{14.5} - \frac{\mathrm{OD}_{558.5} - \mathrm{OD}_{544}}{14.5}\right) + 42,$$

where $\mathrm{OD} = \log(1/R)$ is the optical density, which is a quantitative characteristic of skin absorption, and the subscripts indicate the wavelength in nanometers, at which the optical density is measured.

This approach to calculation of the hemoglobin index makes it possible to quantitatively estimate the hemoglobin content in the skin tissue independently on the degree of its oxygenation, and it is applied, in particular, in studies on the blood content of the skin tissue under mechanical compression [38].

At the second stage of the complex experimental studies, the biotissue fluorescence was assessed using FS and tissue perfusion of blood was estimated by LDF. The perfusion parameters and amplitudes of the biotissue fluorescence were simultaneously recorded in almost the same tissue volume by a LAZMA MC diagnostic complex (SPE LAZMA Ltd., Russia) [39]. The Doppler channel had a laser unit with an emission wavelength of 1064 nm applied to it, and, to excite fluorescence, sources at emission wavelengths of 365 and 450 nm were used. The same fiber-optic probe that had a diameter of 3 mm was used to deliver probing radiation and to collect secondary radiation that was reflected from the tissue. The probing fiber of the LDF channel had a diameter of 6 μm, and the receiving fiber was 400 μm in diameter. The source-to-receiver distance for the LDF channel was 1.5 mm. The FS channel had probing and receiving fibers that were 400 μm in diameter. The source-to-receiver distance was 1 mm. The numerical aperture of the fibers was 0.22. In conducting studies using the data that were obtained in [40], special attention was paid to minimization of the mechanical pressure that was applied to the skin by the probe.

Table 1. Research technique using temperature probes

No.	Stage	Temperature, °C	Time, min
1	Basic test	No normalization	4
2	Local cooling	25	4
3	Local heating	35	4
4	Local heating	42	4

Thermal probes were implemented using a device of a LAZMA-TEST complex (SPE LAZMA Ltd., Russia). In the experimental studies an optical probe was installed on the dorsal surface of foot at a point that was located in the plateau between the first and second metatarsal bones (the control point that is given in Fig. 1). The scheme of the experimental setup for studying hemodynamic parameters and biotissue fluorescence, the location of the optic probe of the LAZMA MC complex, and that of the thermal probe of LAZMA-TEST during the experimental investigations are given in Fig. 2.

Each analysis of the second stage included four periods: a basic test for 4 min, cooling to 25°C for 4 min, and local heating tests for 4 min with temperatures of 35 and 42°C each. Thus, the total duration of the measurement was 16 min (scheme of the experiment is given in Fig. 2). Cooling to 25°C was due to the differences in the initial temperature in the region under study and the necessity to make the initial conditions of the skin temperature in the region under study equal in all the subjects. The sequence of the experimental investigations is given in Table 1.

Amplitudes of fluorescence AF_{365} and AF_{450} that were normalized to the intensity of backreflected excitation radiation and average tissue perfusion by blood I_m at each stage of the functional test were analyzed.

All the parameters that were obtained as a result of processing of the data of the first and second stages of

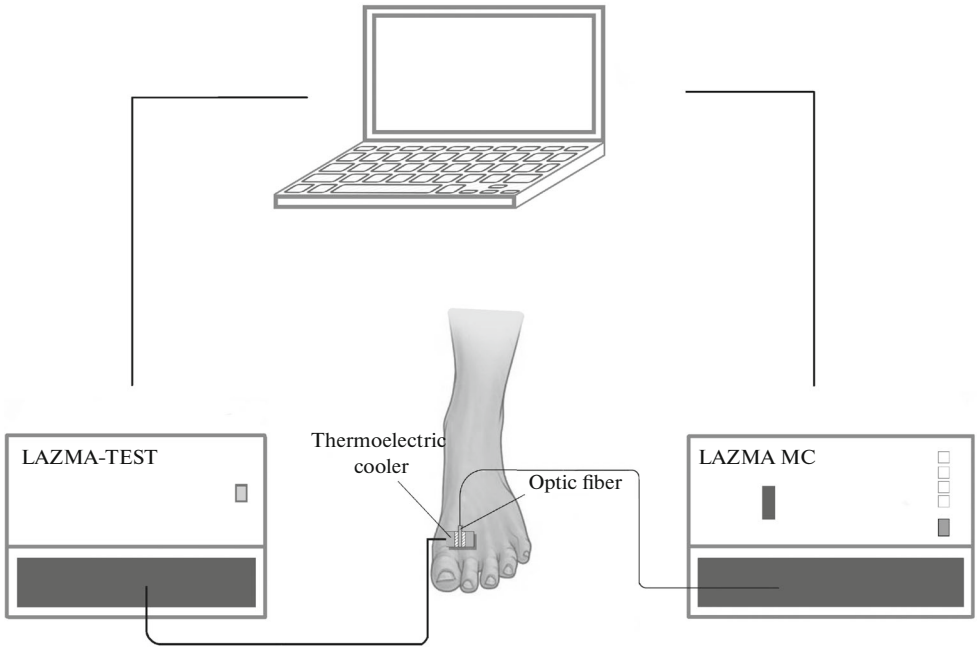

Fig. 2. Scheme of an experimental setup for analysis of the hemodynamic parameters of the fluorescence spectra of skin.

the experiment were tested for normality and homogeneity of variances when compared using the Kolmogorov–Smirnov test and Levene's tests, respectively. The significance of the statistical differences in the samples was assessed by one-way ANOVA. The value $p < 0.01$ was considered significant.

For the second stage of the study, which included analysis of the LDF signals and fluorescence spectra of patients, linear discriminant analysis was used to determine a discriminant function that would make it possible to synthesize a required decision rule and, so, to classify a reappearing object in one of the three groups under analysis on the basis of the measured parameters. The main idea is to determine if different sets differ in the mean of any variable (or linear combination of variables) and then use this variable to predict the belonging of a new object to one or another group. The quality of the discriminant analysis was estimated using an ROC curve.

RESULTS AND DISCUSSION

The first stage of the experimental studies has revealed that the patients with DM that have foot trophic disorders have an elevated hemoglobin index. The statistical significance of the differences in this parameter (Table 2) was confirmed when the data from the spectra that were obtained in the control point were processed and compared with the hemoglobin index in the control group. The hemoglobin index in the control point of the feet of patients without ulcers does not statistically significantly differ from the same parameter that has been recorded in the volunteers of the control group. It can be also seen that the hemoglobin index that has been calculated accord-

Table 2. Results of the calculation of the hemoglobin index and degree of its oxygenation for different foot points in groups under study

Area under study	Ulcer region	Point at 1 cm from ulcer	Control point at the line between the first and second metatarsal bones in patients with ulcers	Control point at the line between the first and second metatarsal bones in patients without ulcers	Control point in volunteers from control group
H	0.47 ± 0.40	0.15 ± 0.08	0.17 ± 0.10*	0.09 ± 0.08	0.07 ± 0.06
Y, %	41.6 ± 4.6	42.0 ± 1.1	41.7 ± 1.4	41.8 ± 6.6	42.5 ± 5.4

* Statistically significant difference in the values of the hemoglobin index as compared to the hemoglobin index for the control group, $p < 0.01$.

Table 3. Results of experimental studies

Parameter	Patients with ulcers	Patients without ulcers	Control group
Normalized amplitude AF_{365}, rel. units	3.8 ± 0.7*(**)	2.7 ± 0.8*	2.1 ± 0.8
Normalized amplitude AF_{450}, rel. units	2.5 ± 0.6*(**)	1.8 ± 0.7*	1.2 ± 0.4
Perfusion I_m, PU	3.8 ± 1.6	5.3 ± 2.2	5.0 ± 1.9
Perfusion I_m, PU at 25°C	3.6 ± 1.4	4.7 ± 2.0	4.4 ± 1.6
Perfusion I_m, PU at 35°C	5.0 ± 1.4*(**)	6.6 ± 2.4*	8.7 ± 3.1
Perfusion I_m, PU at 42°C	9.2 ± 4.6*(**)	12.3 ± 3.5*	19.9 ± 4.6

* Statistical significance of differences, when compared to healthy volunteers, $p < 0.01$.

** Statistical significance of differences, when compared to patients with endocrine disorders without ulcers, $p < 0.01$.

ing to the spectra directly from the ulcers is higher than that in intact regions; however, these differences have not reached statistical significance.

Moreover, for the first stage of the study, the degree of oxygenation of skin hemoglobin was calculated by DRS. There have been no significant differences in the parameter in the groups under study. The results of the calculation of the degree of oxygenation for patients with endocrine disorders and healthy volunteers are given in Table 2. Statistical processing was carried out similarly to the processing of the data for hemoglobin index.

The experimental studies of the second stage by LDF and FS have demonstrated that patients with DM have elevated values of normalized fluorescence amplitudes and weaker perfusion response to local heating from 35 to 42°C. The group with the presence of pronounced complications had significantly different such parameters from the control and general group of patients with diabetes (Table 3, Fig. 3).

The results of the computation of the hemoglobin index in the control point show that high blood filling is observed in patients with focal impairments of the tissue metabolism in their feet. This parameter can be affected by the fact that the groups with trophic disorders included patients with a more prolonged course of diabetes and higher glycated hemoglobin, the optical properties of which could contribute to the diffuse reflectance spectra that were obtained. The hemoglobin index directly characterizes blood filling the skin, and its elevated values that have been measured directly in ulcer regions can be accounted for by a blood flow to these regions due to the inflammation and healing processes, which can be observed in patients with complications of DM. The wide range of this parameter can be explained by different types and stages of the development of trophic ulcers under

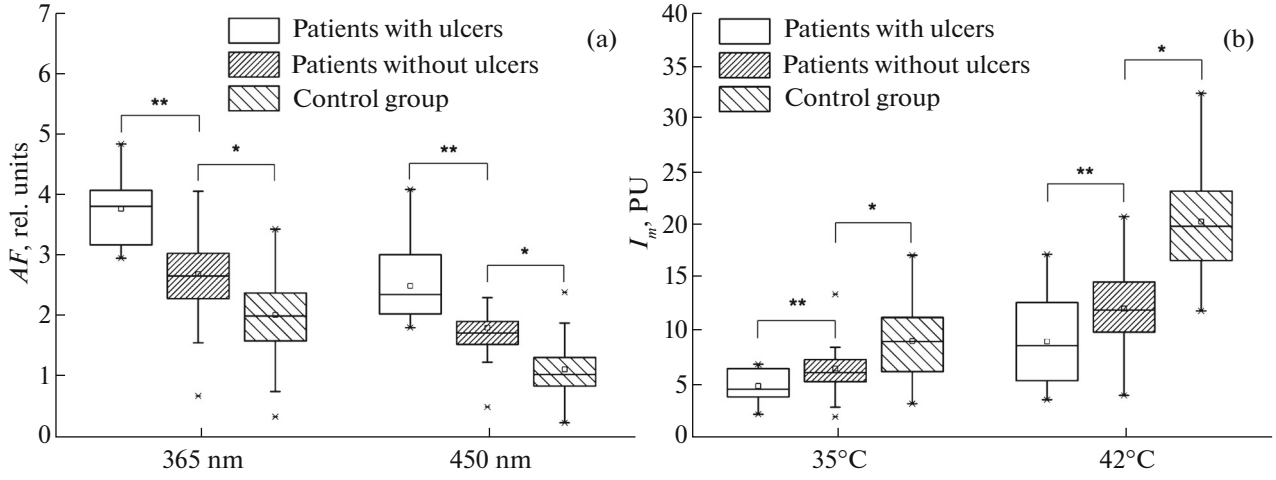

Fig. 3. Results of experimental studies: (a) normalized amplitudes of fluorescence and (b) average perfusion at the stages with heating to 35 and 42°C. *Statistical significance of differences, when compared to healthy volunteers, $p < 0.01$; **statistical significance of differences, when compared to patients with endocrine disorders without ulcers, $p < 0.01$.

study. Studying changes in the hemoglobin index in patients with diabetes can be further used for the development of an approach for evaluations of healing of ulcers and monitoring of the skin response during treatment of patients with diabetes.

The statistically significant differences in the fluorescent signals for the groups under study are explained as follows. As was mentioned, AGEs are involved in the development of diabetes complications. Rapid formation of intracellular AGEs promotes protein dysfunctions, and their accumulation can be an objective marker for advanced glycation in tissues. In addition, a widely accepted index of glycation in tissues, *HbA*1c, describes glycation processes that have occurred for a short period (approximately three months), while changes in the accumulation of AGEs in the skin characterize a more prolonged period. By taking the long lifetime of collagen molecules and stability of AGEs into account, it is possible to use skin fluorescence as an indicator of the integral effect of hyperglycemia over a lifetime.

Diabetes is known to lead to tissue hypoxia [41–43]. In the case of hypoxia, the aerobic respiratory pathway in cells is impaired and mitochondrial oxidation of coenzymes slows down. In connection with this, accumulation of NADH and FAD can serve as a characteristic of tissue hypoxia with its contribution to the total fluorescence signal and be a marker of general oxygen deficiency in tissues. At the same time, changes in the concentrations of NADH and FAD during oxidative phosphorylation are very complicated, and their dynamics can be nonlinear over the entire cell life. This should be considered when interpreting the obtained data and in further studies in this field.

It has also been shown [44–46] that accumulation of AGEs can suppress NO synthesis in endothelial cells. This may also explain the differences in the perfusion response to the stimulation test. Perfusion growth at local thermal probing occurs mainly due to two mechanisms. At skin heating to 34–35°C peptidergic nerve fibers are activated. This is caused by activation of type 1 thermosensitive vanilloid receptors of sensory fibers (TRPV-1) [47]. At skin heating to 42°C, vasodilatation is connected with NO release from endothelial vessels [48, 49]. Topographically, both arterioles and capillaries are involved in the regulatory response to local heating. In diabetes mellitus, dysfunction of all parts of a microcirculatory bed and tissue systems consistently occurs, including of vessel endothelium and perivascular nerve fibers. Thus, a thermal probe is pathogenetically appropriate for diagnostics of these disorders at DM. A decrease in the perfusion growth at skin heating to 35°C is an objective criterion of dysfunction of sensory nerve fibers as a component of diabetic neuropathy. A decrease in the perfusion growth at skin heating to 42°C reflects deficiency in endothelium-dependent mechanisms of vasodilatation.

Thus, skin fluorescence and tissue perfusion by blood upon a thermal probe may be a marker of different degrees of complications from DM onset to the formation of trophic ulcers.

To synthesize a decision rule, we suggest that the analyzed parameters be the normalized amplitudes of skin fluorescence and perfusion that are obtained at the second stage of the experimental investigation. It has been preliminarily established that these parameters are statistically independent and their values, which have been calculated for the groups of patients and control, differ significantly.

Figure 4a gives the results of linear discriminant analysis. Discriminant functions are synthesized so as to provide high sensitivity, provided that they have good specificity. The lowest level of error can be obtained when the fluorescence intensity at 365 nm is combined with the perfusion level upon stimulation of the microcirculation at 42°C. For the first rule of classification for the control group and the group of patients with diabetes without ulcers, the sensitivity and specificity that were obtained were 0.92 and 0.90, respectively. For the second classification rule for patients with diabetes and those with ulcers, they were 0.86 and 0.85, respectively. The direct diagnostic criterion is a classification model in the form of discriminant functions that make it possible to classify the functional state of the area under diagnosis (shown in Fig. 4a).

The fact that the experimental data can be grouped means that the variations in the indices of the impairment of the biotissue (skin fluorescence and perfusion) from the control group are rather reproducible and similar in all the patients.

Figure 4b gives ROC curves that are calculated for the obtained discriminant functions.

To compare the quality of different classification rules, it is convenient to use an integral characteristic, AUC (Area Under Curve), which is the area under the ROC curve. In our case, both for the first classification rule (control group and general group of patients with diabetes) and for the second (general group of patients with diabetes and group of patients with complications), AUC = 0.93. These indices are evidence of a high quality of the classifier that has been proposed.

Pathological changes in the functioning of the microcirculatory bed in patients with DM are determined by microangiopathies, in the development of which chronic hyperglycemia plays a crucial role. FS makes it possible to determine if there has occurred accumulation of glycation products in the biotissue of the patients using the fluorescence of AGEs. In turn, accumulation of glycation products causes endothelial dysfunction by affecting NO synthesis, which can be diagnosed using LDF by determining average tissue perfusion by blood. Reduction in the perfusion in the

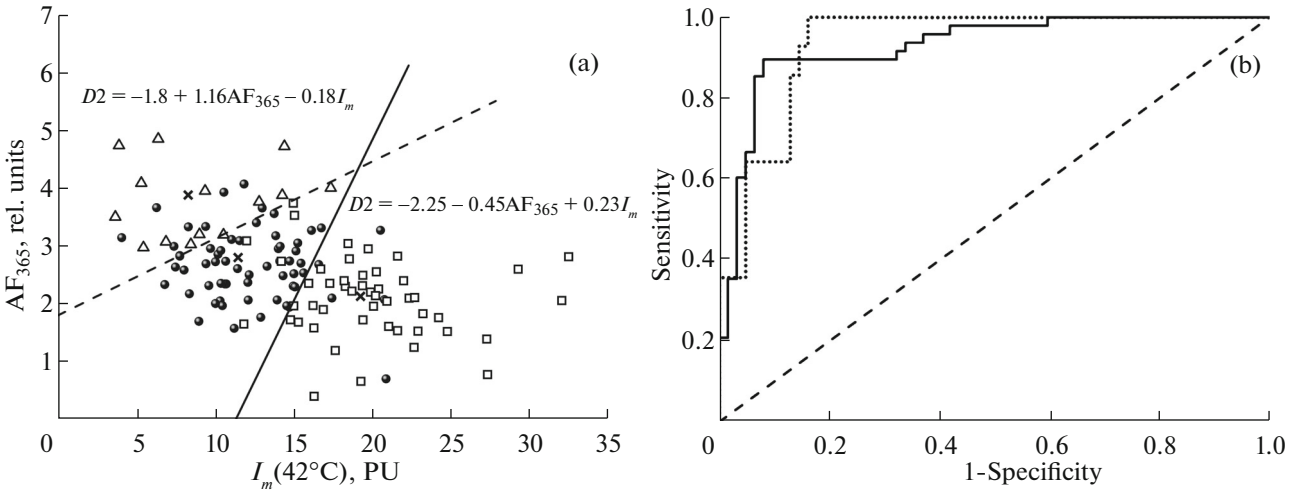

Fig. 4. (a) Results of linear discriminant analysis, $D1 = -2.55 - 0.45AF_{365} + 0.23I_m$ (solid line) and $D2 = -1.8 + 1.16AF_{365} - 0.18I_m$ (dotted line); (b) ROC curves of estimation of the efficacy of the discriminant analysis.

tissues of patients, together with elevated glycated hemoglobin, is likely to affect the hemoglobin index, which can be recorded by DRS. Thus, the results of the processing of combined noninvasive investigations of the foot tissue by spectroscopy make it possible to implement a complex approach for evaluation of microcirculation and tissue impairments in patients with DM.

CONCLUSIONS

The use of diffuse reflectance spectroscopy, laser doppler flowmetry, and fluorescence microscopy in combination or individually can have important clinical applications, since they can help identifying patients with different degrees of microcirculation impairments in the lower extremities.

Hemoglobin makes a significant contribution to the diffuse reflectance spectra of the human skin in the visible and near-infrared ranges. A change in its content under conditions of impairment of the microcirculatory bed of the foot skin due to diabetic microangiopathy can be seen in the reflectance spectra as a change in the absorption and scattering properties of the skin.

The quantitative estimation of the blood content in skin can be made by calculating the hemoglobin index, the use of which makes it possible to objectively assess the skin color and quantitatively estimate blood filling in the region under study in patients with DM; this can be used when the dynamics of ulcer healing is evaluated. The proposed novel method of combined processing of LDF signals and fluorescence spectra of the biotissue was successfully tested in clinical studies and can be used for the diagnostics of microcirculatory disorders in patients' feet.

Thus, diffuse reflectance spectroscopy, LDF, and fluorescence can be used both in combination and individually as additional noninvasive diagnostic techniques in diabetic foot units for long-term observation of high-risk patients together with such conventional methods for detection of complications of DM as foot examination, assessment of neurological status (assessment of vibration perception), and determination of pain, tactile, and temperature sensitivity, etc. The application of the suggested decision rule in the form of a combination of discriminant functions will make it possible for a researcher or clinician not only to determine the presence of complications of diabetes mellitus, but also to evaluate their severity. The methods that have been presented in this study may indicate the danger of microcirculatory disorders and serve for assessment of therapeutic procedures for prevention of the progress of diabetic complications.

ACKNOWLEDGMENTS

This study was supported by a Grant of the President of the Russian Federation for Young Russian Researchers, project no. MK-7168.2016.8, and by the Russian Foundation for Basic Research, project no. 17-41-590560.

REFERENCES

1. I. I. Dedov, Sakharn. Diabet, No. 3, 6 (2010).
2. V. V. Tuchin, *Handbook of Optical Biomedical Diagnostics* (Fizmatlit, Moscow, 2007; Soc. Photo Optical, 2016).
3. A. I. Krupatkin and V. V. Sidorov, *Functional Diagnostics of the State of Microcirculatory–Tissue Systems: Oscillations, Information, Non-Linearity, Handbook* (LIBROKOM, Moscow, 2013) [in Russian].

4. O. N. Bondarenko, N. L. Ayubova, G. R. Galstyan, and I. I. Dedov, Sakhar. Diabet, No. 1, 33 (2013).
5. V. B. Bregovskii, I. A. Karpova, and E. S. Lekseeva, Sakhar. Diabet, No. 3, 49 (2011).
6. N. A. Shor, Khirurgiya, No. 6, 29 (2001).
7. M. B. Antsiferov, G. R. Galstyan, A. Yu. Tokmakova, and I. I. Dedov, Sakhar. Diabet, No. 2, 2 (2001).
8. V. V. Dremin, V. V. Sidorov, A. I. Krupatkin, G. R. Galstyan, I. N. Novikova, A. I. Zherebtsova, E. A. Zherebtsov, A. V. Dunaev, Z. N. Abdulvapova, K. S. Litvinova, I. E. Rafailov, S. G. Sokolovski, and E. U. Rafailov, Proc. SPIE **9698**, 969810 (2017). doi 10.1117/12.2212758
9. E. V. Potapova, V. V. Dremin, E. A. Zherebtsov, I. N. Makovik, A. I. Zherebtsova, A. V. Dunaev, K. V. Podmasteryev, V. V. Sidorov, A. I. Krupatkin, L. S. Khakhicheva, and V. F. Muradyan, Human Physiol. **43**, 222 (2017). doi 0.1134/S036211971702013X
10. N. Sujatha, B. S. Suresh Anand, K. Bala Nivetha, V. B. Narayanamurthy, V. Seshadri, and R. Poddar, J. Appl. Spectrosc. **82**, 432 (2015). doi 10.1007/s10812-015-0125-9
11. A. Nouvong, B. Hoogwerf, E. Mohler, B. Davis, A. Tajaddini, and E. Medenilla, Diabetes Care **32**, 2056 (2009). doi 10.2337/dc08-2246
12. B. S. Suresh Anand, N. Sujatha, V. B. Narayanamurthy, V. Seshadri, and R. Poddar, Opt. Lasers Eng. **53**, 1 (2014). doi 10.1016/j.optlaseng.2013.07.020
13. C. Lal and S. N. Unni, Med. Biol. Eng. Comput. **53**, 557 (2015). doi 10.1007/s11517-015-1266-y
14. A. A. Glazkov, D. A. Kulikov, A. V. Dreval', Yu. A. Kovaleva, V. I. Shumskii, and D. A. Rogatkin, Al'man. Klin. Med., No. 31, 7 (2014).
15. V. V. Dremin, E. A. Zherebtsov, I. N. Makovik, I. O. Kozlov, V. V. Sidorov, A. I. Krupatkin, A. V. Dunaev, I. E. Rafailov, K. S. Litvinova, S. G. Sokolovski, and E. U. Rafailov, Proc. SPIE **10063**, 1006303 (2017). doi 10.1117/12.2252427
16. A. V. Dunaev, E. A. Zherebtsov, D. A. Rogatkin, N. A. Stewart, S. G. Sokolovski, and E. U. Rafailov, Proc. SPIE **8936**, 89360D (2014). doi 10.1117/12.2035651
17. A. I. Zherebtsova, E. A. Zherebtsov, A. V. Dunaev, K. V. Podmasteryev, O. V. Pilipenko, A. I. Krupatkin, L. S. Khakhicheva, and V. F. Muradyan, Proc. SPIE **9917**, 99170M (2016). doi 10.1117/12.2229827
18. S. J. Lin and L. Guarente, Curr. Opin. Cell Biol. **15**, 241 (2003). doi 10.1016/S0955-0674(03)00006-1
19. J. H. Hwang, D. W. Kim, E. J. Jo, Y. K. Kim, Y. S. Jo, J. H. Park, S. K. Yoo, M. K. Park, T. H. Kwak, Y. L. Kho, J. Han, H. S. Choi, S. H. Lee, J. M. Kim, I. Lee, T. Kyung, C. Jang, J. Chung, G. R. Kweon, and M. Shong, Diabetes **58**, 965 (2009). doi 10.2337/db08-1183
20. Y. Ido, C. Kilo, and J. R. Williamson, Diabetologia **40**, S115 (1997). doi 10.1007/s001250051422
21. A. Mayevsky and B. Chance, Mitochondrion. **7**, 330 (2007). doi 10.1016/j.mito.2007.05.001
22. A. Mayevsky and G. G. Rogatsky, AJP Cell Physiol. **292**, C615 (2006). doi 10.1152/ajpcell.00249.2006
23. E. T. Obi-Tabot, L. M. Hanrahan, R. Cachecho, E. R. Beer, S. R. Hopkins, J. C. Chan, J. M. Shapiro, and W. W. LaMorte, J. Surg. Res. **55**, 575 (1993). doi 10.1006/jsre.1993.1187
24. F. Bartolomé and A. Y. Abramov, Methods Mol. Biol. **1264**, 263 (2015). doi 10.1007/978-1-4939-2257-4_23
25. K. A. Foster, C. J. Beaver, and D. A. Turner, Neuroscience **132**, 645 (2005). doi 10.1016/j.neuroscience.2005.01.040
26. I. J. Rampil, L. Litt, and A. Mayevsky, J. Clin. Monit. **8**, 216 (1992). doi 10.1007/BF01616779
27. G. Papayan, N. Petrishchev, and M. Galagudza, Photodiagn. Photodyn. Ther. **11**, 400 (2014). doi 10.1016/j.pdpdt.2014.05.003
28. P. Gkogkolou and M. Böhm, Dermatoendocrinol. **4**, 259 (2012). doi 10.4161/derm.22028
29. R. Meerwaldt, R. Graaff, P. H. N. Oomen, T. P. Links, J. J. Jager, N. L. Alderson, S. R. Thorpe, J. W. Baynes, R. O. B. Gans, and A. J. Smit, Diabetologia **47**, 1324 (2004). doi 10.1007/s00125-004-1451-2
30. K. C. Tan, W. S. Chow, V. H. Ai, C. Metz, R. Bucala, and K. S. Lam, Diabetes Care **25**, 1055 (2002). doi 10.2337/diacare.25.6.1055
31. E. G. Gerrits, H. L. Lutgers, N. Kleefstra, R. Graaff, K. H. Groenier, A. J. Smit, R. O. Gans, and H. J. Bilo, Diabetes Care **31**, 517 (2008). doi 10.2337/dc07-1755
32. D. R. Sell, R. H. Nagaraj, S. K. Grandhee, P. Odetti, A. Lapolla, J. Fogarty, and V. M. Monnier, Diabetes. Metab. Rev. **7**, 239 (1991). doi 10.1002/dmr.5610070404
33. D. R. Sell, A. Lapolla, P. Odetti, J. Fogarty, and V. M. Monnier, Diabetes **41**, 1286 (1992). doi 10.2337/diab.41.10.1286
34. M. Takahashi, H. Hoshino, K. Kushida, and T. Inoue, Anal. Biochem. **232**, 158 (1995). doi 10.1006/abio.1995.0002
35. E. A. Zherebtsov, A. V. Bykov, A. P. Popov, A. Doronin, and I. V. Meglinski, Proc. SPIE **10063**, 1006312 (2017). doi 10.1117/12.2250855
36. J. W. Feather, Hajizadeh-M. Saffar, G. Leslie, and J. B. Dawson, Phys. Med. Biol. **34**, 807 (1989). doi 10.1088/0031-9155/34/7/002
37. M. Hajizadeh-Saffar, J. W. Feather, and J. B. Dawson, Phys. Med. Biol. **35**, 1301 (1990). doi 10.1088/0031-9155/35/9/009
38. O. A. Zyuryukina, I. A. Nakhaeva, R. Mokhammod, and Yu. P. Sinichkin, in *Proceedings of the 17th International Young Scientists School on Problems of Optical Physics and Biophotonics SFM-2013,* p. 9.
39. V. V. Dremin, E. A. Zherebtsov, I. E. Rafailov, A. Y. Vinokurov, I. N. Novikova, A. I. Zherebtsova, K. S. Litvinova, and A. V. Dunaev, Proc. SPIE **9917**, 99170Y (2016). doi 10.1117/12.2229451
40. E. A. Zherebtsov, K. Y. Kandurova, E. S. Seryogina, I. O. Kozlov, V. V. Dremin, A. I. Zherebtsova, A. V. Dunaev, and I. V. Meglinski, Proc. SPIE **10336**, 1033608 (2017). doi 10.1117/12.2267957
41. L. Xi, C.-M. Chow, and X. Kong, J. Diabetes Res. Hindawi **2016**, 1 (2016). doi 10.1155/2016/1527852

42. H. Thangarajah, D. Yao, E. I. Chang, Y. Shi, L. Jazayeri, I. N. Vial, R. D. Galiano, X. L. Du, R. Grogan, M. G. Galvez, M. Januszyk, M. Brownlee, and G. C. Gurtner, Proc. Natl. Acad. Sci. **106**, 13505 (2009). doi 10.1073/pnas.0906670106

43. J. Ditzel and E. Standl, Acta Med. Scand. Suppl. **578**, 59 (1975). doi 10.1111/j.0954-6820.1975.tb06503.x

44. R. Bucala, K. J. Tracey, and A. Cerami, J. Clin. Invest. **87**, 432 (1991). doi 10.1172/JCI115014

45. A. W. Stitt, C. He, S. Friedman, L. Scher, P. Rossi, L. Ong, H. Founds, Y. M. Li, R. Bucala, and H. Vlassara, Mol. Med. **3**, 617 (1997).

46. U. Chakravarthy, R. G. Hayes, W. Stitt, E. McAuley, and D. B. Archer, Diabetes **47**, 945 (1998). doi 10.2337/diabetes.47.6.945

47. D. P. Stephens, N. Charkoudian, J. M. Benevento, J. M. Johnson, and J. L. Saumet, Am. J. Physiol. Regul. Integr. Comp. Physiol. **281**, R894 (2001).

48. C. T. Minson, L. T. Berry, and M. J. Joyner, J. Appl. Physiol. **91**, 1619 (2001).

49. D. L. Kellogg, J. L. Zhao, Y. Wu, and Y. Wu, J. Appl. Physiol. Am. Physiol. Soc. **107**, 1438 (2009). doi 10.1152/japplphysiol.00690.2009

Translated by E. Berezhnaya