ORIGINAL ARTICLES

LASER FLUORESCENT SPECTROSCOPY AND OPTICAL TISSUE OXIMETRY IN DIAGNOSTICS OF SKIN FIBROSIS

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Abstract

There are currently no effective measures to combat fibrosis in modern medical practice. One of the reasons for that is the late diagnosis associated with the lack of available clinical biomarkers and effective methods of non-invasive detection of the process. Fibrosis of the skin is characterized by fibrosis of the dermis, underlying tissues and is represented by a wide range of nosologies. Scleroderma and scars are of the greatest interest for the study. Skin changes in the development of bleomycin-induced fibrosis was studied in the experimental model using laser fluorescence spectroscopy and optical tissue oximetry. A significant increase in the rates of endogenous fluorescence of porphyrins, caused by inflammation and hypoxia, was detected at 7 and 21 days. An increased intensity of endogenous collagen fluorescence and a decreased specific oxygen uptake due to excess accumulation of the extracellular matrix were recorded on the 21st day after bleomycin treatment. Synchronous measurements of the collagen fluorescence and the specific oxygen uptake allowed to correlate the obtained data and the phases of the fibrogenic response described morphologically. The results allow to judge the severity of inflammation and hypoxia in the process of the fibrosis development. The objective and quantitative nature of the recorded parameters makes it possible to develop criteria for diagnosing the phases of fibrosis development.

Keywords: fibrosis, laser fluorescence spectroscopy, optical tissue oximetry, diagnostics in vivo

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ЛАЗЕРНАЯ ФЛУОРЕСЦЕНТНАЯ СПЕКТРОСКОПИЯ И ОПТИЧЕСКАЯ ТКАНЕВАЯ ОКСИМЕТРИЯ В ДИАГНОСТИКЕ ФИБРОЗА КОЖИ

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Резюме

В современной медицинской практике нет эффективных мер борьбы с фиброзом. Одна из причин – поздняя диагностика, связанная с отсутствием доступных клинических биомаркеров и эффективных методов неинвазивного обнаружения этого процесса. Фиброзирующие заболевания кожи характеризуются фиброзом дермы, подлежащих тканей и представлены широким спектром нозологий. Наибольший интерес для изучения представляют склеродермия и рубцы кожи. На экспериментальной модели методами лазерной флуоресцентной спектроскопии и оптической тканевой оксиметрии изучены изменения кожи в рамках развития блеомицин-индуцированного фиброза. Выявлен достоверный рост показателей эндогенной флуоресценции порфиринов на 7 и на 21 сут, вызванный воспалением и гипоксией. Зафиксированы повышение интенсивности эндогенной флуоресценции коллагена и снижение показателей удельного потребления кислорода на 21 сут исследования, связанные с избыточным накоплением межклеточного матрикса. Синхронные измерения флуоресценции коллагена и удельного потребления кислорода позволили провести корреляцию с фазами фиброгенного ответа, описанного морфологически. Полученные результаты позволяют судить о выраженности воспаления и гипоксии в процессе развития фиброза. Объективный и количественный характер регистрируемых параметров дает возможность разработки критериев для диагностики фаз развития фиброза.

Ключевые слова: фиброз, лазерная флуоресцентная спектроскопия, оптическая тканевая оксиметрия, диагностика *in vivo*.

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Introduction

Systemic and organ fibrosis are some of the serious medical problems affecting a significant proportion of the world's population [1]. Fibrosis is a leading process in the development of autoimmune conditions, such as rheumatoid arthritis, Crohn's disease, ulcerative colitis, systemic lupus erythematosus, as well as in diseases of the liver, kidneys, pulmonary alveolitis and heart failure [2].

In the Russian medical academic tradition, an excessive accumulation of connective tissue in the skin is commonly referred to as sclerosis, which is the final stage of tissue fibrosis, with the loss of functions of the organ [3]. The term «fibrosis» in modern periodicals is more and more often used to refer to accumulation and disorganization of connective tissue [4].

Skin fibrosis is most often manifested as scleroderma, hypertrophic and keloid scars [5]. Fibrous changes in this case can have a different degree of severity: from cosmetic defects in the case of cicatricial deformity to life-threatening conditions in the case of systemic scleroderma [6–8]. The pathogenesis of skin fibrosis in different diseases has similar features and is characterized by proliferation of fibroblasts, myofibroblasts, excessive synthesis and accumulation of connective tissue [9]. Fibroblast activation is always a key link in skin fibrosis [10]. It is known that their uncontrolled proliferation can be caused by chronic inflammation, infection, autoimmune and allergic reactions, as well as damage to the skin due to radiation or chemical exposure. Persistent activation of fibroblasts in this case promotes excessive synthesis of intercellular substance which mainly consists of collagen, elastin, non-collagen glycoprotein and proteoglycan [11]. Excessive fiber synthesis and deposition of the intercellular matrix result in skin fibrosis.

There is a point of view discussed in the scientific literature that describes fibrosis not as the outcome of tissue damage, but as a dynamically progressing and reversible process associated with inflammation and hypoxia [12, 13], so that timely intervention enhances the therapeutic options [14]. Thus, the justified choice of the treatment method for the developing hypertrophic and keloid scars is based on the understanding of the prevailing process involved (inflammation/hypoxia/fibrosis). Studies in this area show that the response of fibrosed tissues to a particular type of treatment depends on the adequacy of the current treatment factor to the nature of the pathological process that determines the functional state of the tissue [15]. During the examination, a clinician may not objectively determine the activity and contribution of individual processes (inflammation/hypoxia/fibrosis). Histological examination makes it possible to get more of objective information, however, the process of collecting biological material (biopsy) can cause subsequent excessive growth of the scar [16]. Today, there are no generally accepted algorithms for choosing a method for scar treatment, and the tactics of managing a particular patient are based on the personal experience of the doctor and the traditions of individual clinical schools and organizations [17].

The analysis of modern research has shown that non-invasive methods for diagnosing skin fibrosis, such as ultrasound, elastography, confocal microscopy, optical coherence tomography, still have not become wide-spread in everyday medical practice. First of all, this is due to the lack of criteria that reliably characterize fibrosis [18]. Therefore, we can definitely say that the task of developing a method for rapid, non-invasive, quantitative assessment of fibrosis remains relevant for medicine. We believe that optical technologies can have diagnostic potential, which may provide the basis for a fundamentally new approach to an integrated assessment of this process. For example, it is known that excess collagen can be detected by laser fluorescence spectroscopy, since this substance fluoresces under the influence of light in the UV range [19], and fluorophores responsible for inflammation and hypoxia can also be detected in the red and green spectra [20]. Optical tissue oximetry methods make it possible to determine the specific oxygen consumption of tissues, which characterizes the activity of proliferative processes. Therefore, it can be presumed that the results of laser fluorescence spectroscopy and optical tissue oximetry taken together could help determine the leading pathological process, which will allow a personalized approach to the selection of therapy.

The purpose of our work was to study the diagnostic capabilities of optical methods of laser fluorescence spectroscopy and optical tissue oximetry in assessing fibrosis in animal skin.

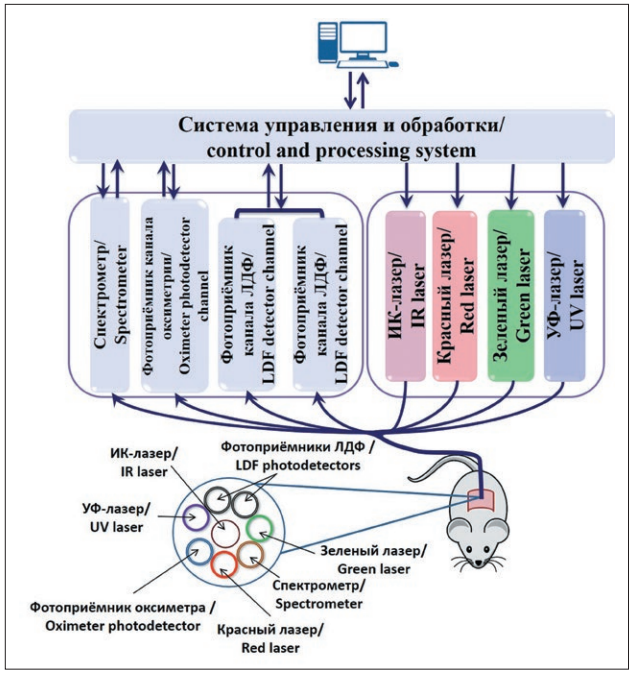

Рис. 1. Схема экспериментальной установки и расположение осветительных и приёмных волокон в оптоволоконном жгуте
Fig. 1. Experimental setup and location of illuminating and receiving optical fibers in the probe

Materials and methods

The study was conducted on outbred white mice, males, aged 6 weeks, weighing 25-30 g, number of animals (N): 47. The animals were kept in standard vivarium conditions at a temperature of 21–23°C, humidity of 50–65%, and 14-hour long daylight. They received a balanced granular food which contained no fluorophores and had unlimited access to drinking water.

The experiment was carried out in compliance with the principles of the Declaration of Helsinki on the Humane Treatment of Animals, the principles of humanity set forth in the European Community Directive (86/609/EC), the European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (ETS 123) Strasbourg, 1986.

Fibrosis was created with the use of the relevant model of skin fibrosis in animals, which is used to study scleroderma and cicatricial changes in the skin [21, 22]. The animals were divided into 2 groups. The first group (N = 30) was administered subcutaneous injections of bleomycin (BLM) at a dosage of 0.1 ml (concentration 0.5 mg/ml). The second (control) group (N = 17) was administered subcutaneous injections of 0.1 ml of 0.9% NaCl (PBS). All animals were injected daily for 21 days into the previously depilated skin of the interscapular region of the back. The first four injections were administered at the vertices of a 1 cm^2 square previously marked with a marker, and the fifth was made in the center of the square.

On days 0, 7, 14, and 21, the intensity of endogenous fluorescence, tissue saturation of oxyhemoglobin, and volumetric blood filling of the skin *in vivo* were measured. Indications were taken from the skin surface directly above the experimental site (Fig. 1). All measurements were performed with a multifunctional laser diagnostic complex «LAKK-M» (OOO RPE «LAZMA», Russia) [23].

Fig. 1 shows a schematic diagram of «LAKK-M» complex. The complex operates in «fluorescence» and «microcirculation» modes. In the «microcirculation» mode, the complex continuously measures the hemoglobin oxygen saturation and the volume of the hemoglobin fraction at the site subject to probing. These indicators are calculated according to the methodology of absorption spectroscopy, which is based on the difference in the recorded signals when probing biological tissue in the red and green spectral wavelength ranges. Hemoglobin oxygen saturation is determined on the basis of different optical properties of the oxygenated and deoxygenated hemoglobin fractions contained in the diagnostic blood volume. Based on these indicators averaged over the measurement time (15 s), the specific oxygen consumption by cells (U) was calculated, which characterizes the oxygen consumption per unit volume of blood circulating in the blood tissue according to the formula [24]:

$$U = \frac{(S_pO_2 - S_tO_2)}{V_b},$$

where S_tO_2 is the average tissue saturation of oxyhemoglobin, V_b is the average volumetric blood supply. At the same time, the saturation of oxyhemoglobin (S_pO_2) in arterial blood was assumed to be 98%.

The «fluorescence» operating mode is used to implement the method of laser fluorescence spectroscopy. Radiation from the selected source is delivered to the surface of the test volume with the use of a fiber optic probe through a lighting fiber. Secondary radiation is delivered to the spectrometer through the receiving fiber.

To excite fluorescence in various parts of the spectrum, low-power lasers with wavelengths λ_e = 365 and 535 nm were used. The output power at the distal end of the fiber optic probe is about 2-3 mW for each light source. The wavelengths at which the fluorescence of the studied fluorophores reaches the values that are most effective for recording are hereinafter denoted by λ_f. For collagen, λ_f = 445–455 nm, for porphyrin, λ_f = 600–620 nm [19]. It should be noted that the contributions of collagen and elastin to the total spectrum are difficult to separate, therefore, it was further considered that fluorescence in the wavelength range λ_f = 445–455 nm reflects the presence of both fluorophores. In this study, we estimated the dynamics of the intensity at given wavelengths (hereinafter referred to as «fluorescence intensity») with equal laser powers that were tracked.

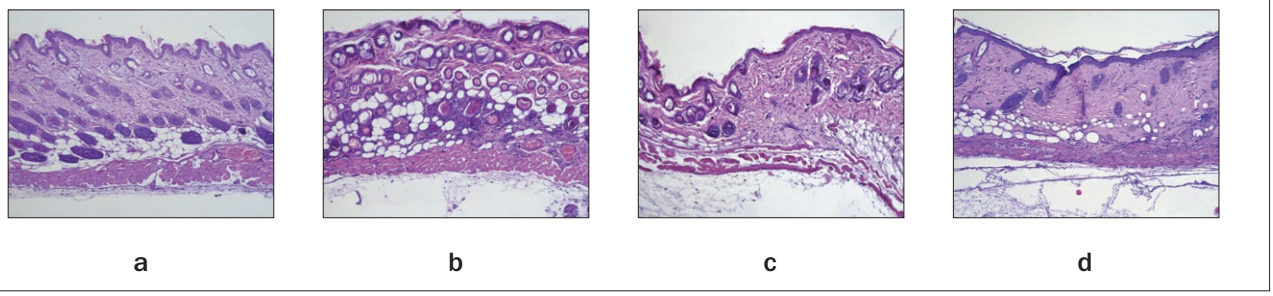

Рис. 2. Кожа мышей в группе BLM. Гистологические препараты, окраска гематоксилином и эозином (увеличение ×100):
a – 0 сут, структура эпидермиса и дермы не изменена;
b – 7 сут, воспалительная инфильтрация долек жировой ткани лимфоцитами и гистиоцитами;
c – 14 сут, частичное замещение жировой ткани межклеточным матриксом, уменьшение воспалительной инфильтрации;
d – 21 сут, обширные очаги накопления межклеточного матрикса, обедненные клеточными элементами (гипоцеллюлярный фиброз), сглаженность коллагеновых волокон

Fig. 2. The skin of mice in the BLM group. Histological preparations, hematoxylin and eosin staining (magnification ×100):
a – Day 0: the structure of epidermis and dermis is not changed;
b – Day 7: inflammation in the lobule of adipose tissue due to infiltration of lymphocytes and histiocytes;
c – Day 14: partial replacement of the adipose tissue by extracellular matrix, reduction of inflammatory infiltration;
d – Day 21: extensive foci of the extracellular matrix deposition, depleted of cellular elements (hypocellular fibrosis), smoothness of collagen fibers

Samples for histological studies were taken on days 0, 7, 14 and 21. From the study area, skin fragments of 1.0 × 1.0 cm were isolated, after which the histological preparations were stained with hematoxylin-eosin. The study of the morphological picture involved the evaluation of the state of the epidermis, the inflammatory changes in the dermis, subcutaneous fat and the structure of collagen fibers.

Statistical analysis was performed with the use of Microsoft Excel (Microsoft Corp., USA). The hypotheses about the presence of differences between groups were checked by comparing the arithmetic mean values and constructing 95% confidence intervals for the arithmetic mean values.

Results and discussion

During the experiment, skin fibrosis confirmed histologically was reproduced in the BLM group of animals (Fig. 2).

Fig. 4a shows the dynamics of the group-averaged fluorescence intensity of collagen and elastin. We believe that a decrease in the intensity of endogenous collagen fluorescence on day 7 compared to day 0 in the BLM group is most likely due to tissue edema due to inflammatory exudation, which was histologically most pronounced at that time. An increase in the intensity of endogenous fluorescence of collagen and elastin on day 21 in the BLM group is caused by their accumulation in

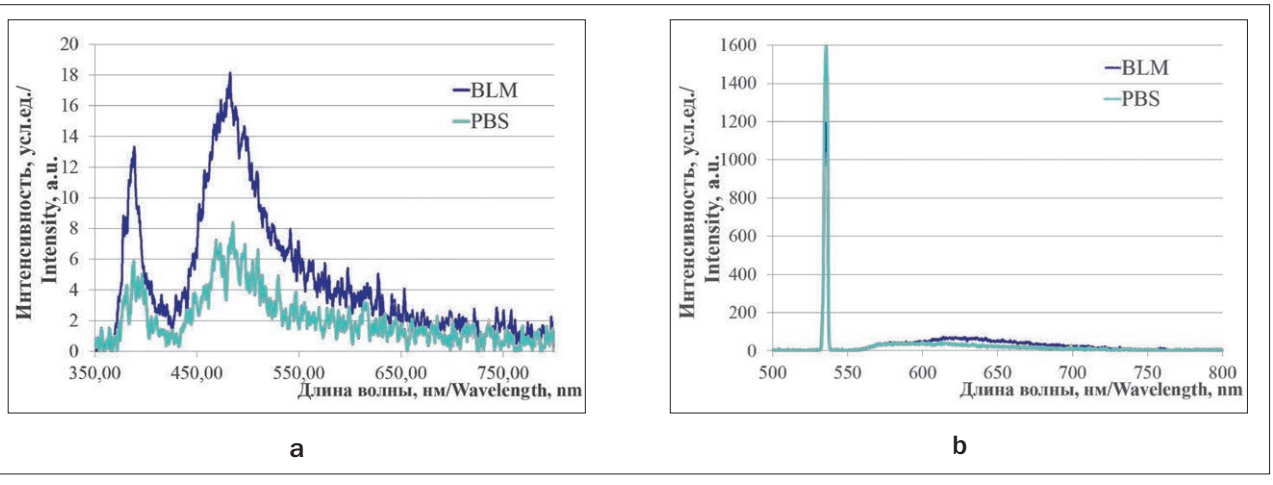

Рис. 3. Примеры спектров флуоресценции в области инъекций на 21 сут:
a – в УФ-диапазоне (λ_e = 365 нм);
b – в зеленом диапазоне (λ_e = 535 нм)

Fig. 3. Example of the fluorescence spectra at the injection site at day 21:
a – in the UV wavelength range (λ_e = 365 nm);
b – in the green wavelength range (λ_e = 535 nm)

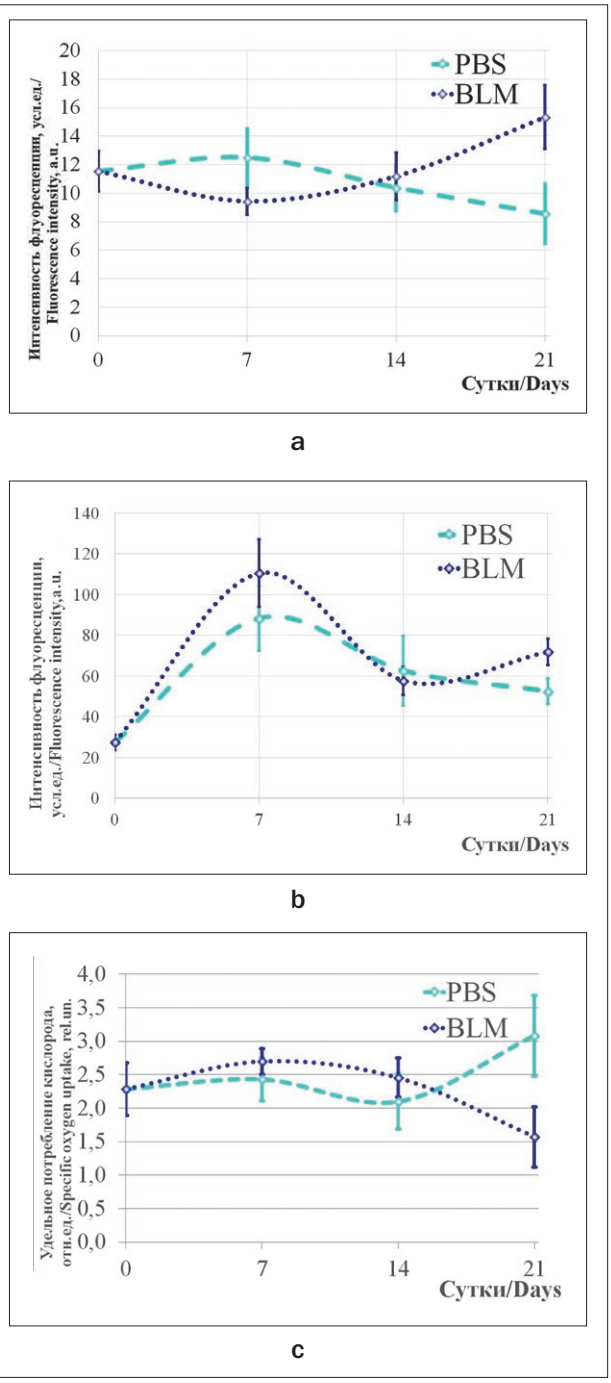

Рис. 4. Динамика показателей оптической диагностики в группах BLM (инъекция блеомицина) и PBS (инъекция физраствора):
a – интенсивности флуоресценции коллагена и эластина (λ_e = 365 нм, λ_f = 445–455 нм);
b – интенсивности флуоресценции порфирина (λ_e = 535 нм, λ_f = 610 нм);
c – удельного потребления кислорода тканями

Fig.4. Dynamics of optical diagnostic indicators in the BLM (bleomycin injection) and PBS (saline injection) groups:
a – fluorescence intensity of collagen and elastin (λ_e = 365 nm, λ_f = 455 nm);
b – fluorescence intensity of porphyrin (λ_e = 535 nm, λ_f = 610 nm);
c – specific oxygen uptake by tissues

the region of the formed fibrosis, which also corresponds to the morphological picture of skin fibrosis. As collagen is the main extracellular substance of connective tissue in case of skin fibrosis [25], the contribution of elastin fluorescence in this case is insignificant.

Porphyrins have been found to rapidly respond to metabolic changes in tissues. In particular, their synthesis is actively increased in cells in a state of chronic hypoxia and inflammation [26]. The dynamics of porphyrin fluorescence averaged over intensity groups (Fig. 4b) shows a significant increase in parameters by 7 days compared to day 0 of the experiment in both groups of animals, which probably reflects the inflammatory processes caused by daily subcutaneous injections. In addition, significant differences in the BLM and PBS groups were obtained on day 21 of the experiment. We believe that an increase in the intensity of endogenous fluorescence of porphyrins in skin with fibrosis during this period is caused by hypoxia, which occurred primarily due to impaired perfusion in tissues [27].

The results of measuring specific oxygen consumption show a significant decrease in its indices by day 21 in the BLM group (Fig. 4c), presumably due to the formation of hypocellular fibrosis with low metabolic activity.

It is reliably known that inflamed and proliferating tissue has a high oxygen demand. This is most pronounced in the structure of immature metabolically active scar tissues. However, when the fibrosis has established, this demand decreases, including due to the reduction in the number of cellular elements [28]. In our experiment, we confirmed this histologically, and this dependence was shown by the results of the calculation of the specific oxygen consumption by the cells and the endogenous fluorescence of collagen on days 14 and 21 in the BLM group (Fig. 5).

The modern concept allows us to divide the fibrogenic response into four overlapping phases: phase 1: the initiation of the response caused by primary damage, phase 2: activation of effector cells, phase 3: production of the extracellular matrix, phase 4: dynamic deposition/insufficient resorption of the extracellular matrix [4]. The results of measuring collagen fluorescence and specific oxygen consumption in combination with the morphological picture of the skin allow us to correlate them with the phases of the fibrogenic response. Thus, in phases 1 and 2 (0–7 days), an increase in oxygen consumption by effector cells is observed due to primary tissue damage and subsequent inflammation and hypoxia of consumption. In phase 3 (7-14 days), collagen fluorescence increases, as evidenced by the accumulation of the intercellular matrix, and oxygen consumption decreases, which is confirmed by the weakening of the inflammation process. Phase 4 (14–21 days) is characterized by excessive accumulation of the intercellular matrix deficient in cellular elements and a sharp drop in oxygen

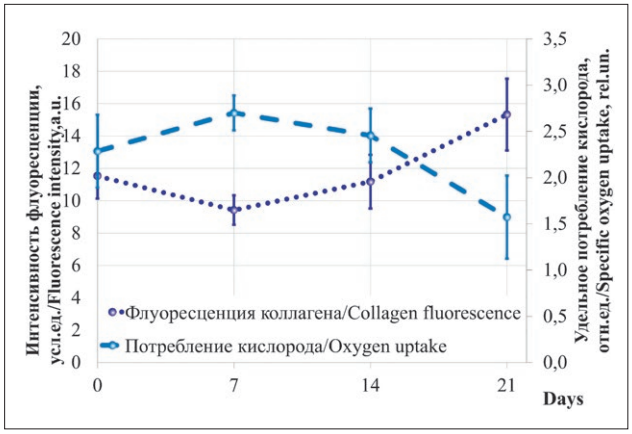

Рис. 5. Динамика интенсивности флуоресценции коллагена и эластина (λ_e = 365 нм, λ_f = 445–455 нм) и удельного потребления кислорода тканями.
Fig. 5. Dynamics of collagen and elastin fluorescence intensity (λ_e = 365 nm, λ_f = 445–455 nm) and specific oxygen uptake by tissues

consumption. However, it is worth noting that the time boundaries at this stage are conventional due to the characteristics of the experiment.

A number of researchers believe that fibrosis is irreversible if the tissue becomes paucicellular and, as a result, poor in biologically active molecules necessary for the degradation of the extracellular substance of connective tissue [29, 30]. This corresponds to the period when an increase in the specific consumption of oxygen is replaced by its decline due to a decrease in the number of cells consuming it. At the same time, an increase in the fluorescence of collagen, as the main biomarker of fibrosis, is recorded. We believe that the simultaneous measurement of collagen fluorescence and specific oxygen consumption will allow us to record not only the synchronous nature of the processes of inflammation and hypoxia involved in fibrogenesis, but also provide new opportunities for the diagnosis of fibrosis phases.

Conclusion

The results of the study demonstrated the possibility of objective non-invasive dynamic monitoring of inflammation and hypoxia in the development of skin fibrosis with the use of laser fluorescence spectroscopy and optical tissue oximetry. The development of the proposed approaches can be implemented by establishing quantitative criteria for a clear periodization of fibrosis formation. The objectivity of the study can also be enhanced through the use of additional methods that simultaneously evaluate and compare the manifestations of the processes under study at the molecular, cellular and tissue levels. This, in turn, will expand the capabilities of practitioners in diagnosing fibrosis, personalizing treatment tactics, and predicting the outcome of the disease.

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