Optical Technology for Fibrotic Skin Changes Objectification in Experimental Systemic Scleroderma

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Abstract: Currently the examination of skin fibrosis is based on subjective non quantitative methods and requires invasive procedures. Optical techniques abled to evaluate different quantitative parameters of ordered tissues can be used to solve these problem. Measurements of endogenous fluorescence intensity, regional tissue oxyhemoglobin saturation, and blood filling volume allowed to define the high endogenous fluorescence intensity of porphyrin in skin fibrosis. Besides that, the decrease in oxygen intake parameters together with the fluorescence intensity increase of collagen was determined. Consequently the optical diagnostic techniques can become an effective method for skin fibrosis evaluation.

1 INTRODUCTION

Fibrosis of different organs and systems is one of severe medical issues as it affects a significant proportion of the human population (Wermuth, 2015; Rockey et al., 2015). It is the main pathological process not only in such autoimmune disorders as scleroderma, rheumatoid arthritis, Crohn's disease, ulcerative colitis, and systemic lupus erythematosus (Wynn et al., 2011), but also in liver, kidney, lung diseases and heart failure (Bataller et al., 2005; Wynn, 2008).

Currently there are a lot of studies on fibrosis. The pathogenesis of tissue fibrosis is considered to be a dynamic and reversible process connected with inflammation and hypoxia (Driskell et al., 2013; Manresa et al, 2014). Nevertheless, the histological examination still remains the reference method for diagnosis, which invasiveness is the main disadvantage as it impairs the examined tissues state (Monstrey et al., 2008).

Fibrotic skin changes are the defining features of all scleroderma forms (Gabrielli et al., 2009). The degree and the rate of fibrosis progression correlate with patients' death rate (Clements et al., 2000; Khanna et al., 2010). In clinical practice the modified Rodnan skin score (mRSS) that measures the skinfold thickness is widely used for skin fibrosis evaluation. However, the information received when using this method is rather subjective. The application of mRSS is essentially limited as it requires special knowledge and skills from physicians. Subcutaneous fat changes developed in some patients suffering from this disease can also lead to diagnostic pitfall. Furthermore skin score is insensitive to initial presentation of a disease that is however clinically significant (Maurer et al., 2014). Non-invasive methods of skin fibrosis diagnosis such as ultrasound scan, elastography, confocal microscopy, and optical coherence tomography are still of limited use in routine clinical practice due to the lack of the accurate criteria for fibrosis assessment (Kang et al., 2014). The microvascular damage dominates in the pathogenesis of skin fibrosis. That causes endothelial cell activation leading to the hypoxia and the increase in the amount of inflammation triggers that starts uncontrolled inflammation response. As a result of it fibroblasts excessively differentiate into myofibroblasts, responsible for extracellular matrix synthesis, which major component is collagen (Jinnin, 2010; Hinz B. et al., 2012; Ho et al., 2014). The excess of collagen is known to be detected by laser fluorescence spectroscopy as this substance fluoresces under UV light (Smirnova et al., 2012).

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Fluorophores responsible for inflammation and hypoxia can also be detected in red and green spectrum range (Franco et al., 2016).

In modern medicine the development of the rapid and non-invasive method enabled to give a quantitative assessment of skin fibrosis is absolutely necessary. In such a case optical techniques have a diagnostic potential to become the basis for a fundamentally new approach to fibrosis comprehensive assessment.

The aim of our study was to examine diagnostic capabilities of optical technologies in animal skin fibrosis evaluation. Animal models are still of a great importance for skin fibrosis pathogenesis investigation, and the results obtained can either be reproduced in clinical researches or give a meaningful data for understanding the pathogenesis of this process (Avouac et al., 2013; De Langhe, 2015).

2 MATERIALS AND METHODS

The study was performed on the outbred white male mice aged 6 weeks with a mass of 25-30 grams, N = 47. Animals were kept in vivarium standard conditions in a 14 hour natural light at a temperature of 21-23 °C and a humidity of 50 - 65%. They received balanced granulated feed, that didn't include fluorophores and had a free water access.

The experiment was conducted in compliance with the welfare of animals used in experiment (Declaration of Helsinki), EU Directive 86/609/EEC on the protection of animals used in experiments, and European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (ETS 123) Strasbourg, 1986).

The relevant animal model of scleroderma was used for fibrosis development. We chose bleomycin-induced fibrosis as it allows to represent the initial presentation of this disease (Avouac, 2014).

Animals were divided into two groups. In the first one (N = 30) subcutaneous injections of bleomycin (BLM) in a dose of 0,1 ml (100μL of bleomycin preliminary dissolved in 0.9% NaCl, concentration 0.5 mg/ml) were administered. In the second one (control group, N = 17) subcutaneous injections of 0,1 ml of 0,9 % NaCl (PBS) were administered. All animals were daily injected in shaved skin of interscapular region during 21 days. The first four injections were made in different angles of a marked square with a size of 1 cm², the fifth one was done in its middle.

On the 0, 7, 14 and 21 day the endogenous fluorescence intensity, regional tissue oxyhemoglobin saturation, and blood filling volume were measured *in vivo*, on the skin surface just above the experimental area. All measurements were taken using non-invasive multifunctional laser diagnostic system "LAKK-M" (SPE 'LAZMA' Ltd, Russia) (Figure 1) on the operating regimes "Fluorescence" and "Microcirculation" (Rogatkin et al., 2009). Scheme of diagnostic system, the mouse locations in the setup and sensor localization are presented at figure 2.

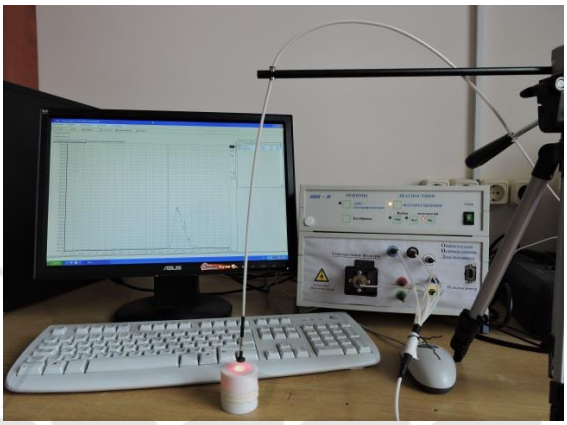

Figure 1: Diagnostic system "LAKK-M".

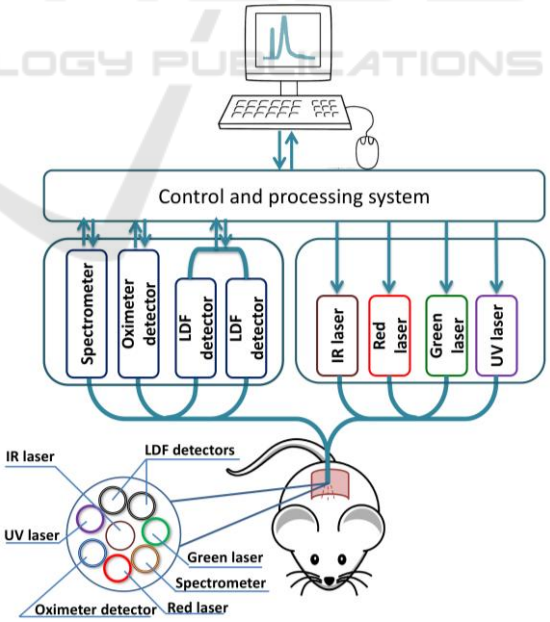

Figure 2: Scheme of the diagnostic system.

Low-power lasers with a wave length of λ_e = 365, 535 and 635 nm were used for fluorescence excitation. The output power at the distal end of the

fiber-optical probe was about 2 – 3 mW for each light source. The wavelengths on which the fluorescence had a maximum value were marked with λ_f. Thus for collagen λ_f = 455 nm, for porphyrin λ_f = 610 nm. It should be noted, that it is hard to separate the fluorescence of collagen and elastin, so in the following we considered that the fluorescence on the wave length of λ_f = 455 nm represents both of fluorophores. In this study the intensity dynamics on this wavelength (later on – "the fluorescence intensity") in controlled equivalent intensity of irradiation was evaluated.

In "Microcirculation" operating regime laser Doppler flowmetry and tissue reflectance oximetry were used enabling to continuously register tissue saturation and blood filling volume values in percent.

The relative oxygen consumption rate (U) characterized by the oxygen intake per tissue blood flow volume unit was assessed according to the time-averaged (15 s) measurements using the following formula (Rogatkin et al., 2013):

$$U = (S_pO_2 - S_tO_2)/ V_b \quad (1)$$

S_tO_2, means tissue oxyhemoglobin saturation, V_b, means blood filling volume. In this case, S_pO_2 is the functional pulse saturation of the oxyhemoglobin fraction in the arterial peripheral blood. It was assumed equal to 98%.

Histological samples were taken on 0, 7, 14, and 21 day. Skin fragments 1,0 cm × 1,0 cm in size were separated from the research region followed by material examination according to a standard protocol. An epidermis condition, inflammatory changes in dermis, subcutaneous fat, and panniculus carnosus, as well as dermis thickness and collagen fibers structure were assessed.

Furthermore, a noncompetitive enzyme-linked immunosorbent assay method was used to evaluate C-reactive protein in control period. For this purpose we used a mice blood serum having been centrifuged in 1500 g mode during 15 minutes. Researches were performed on microplate photometer for enzyme immunoassay Stat Fax 2100, Awareness Technology, USA. We also used Mouse CRP (an enzyme immunoassay kit for the quantitative measurement of mouse CRP), Czech Republic.

Statistical analysis was carried out in Microsoft Excel 2016. A hypothesis for the difference between two groups was tested by the comparison of arithmetic means and the construction of 95% confidence intervals for them.

3 RESULTS AND DISCUSSION

In BLM animal group a skin fibrosis was reproduced by the 21st day of experiment. The histological pattern of tissue in the injection area in both groups on the 21st day of experiment is shown in the Figure 3.

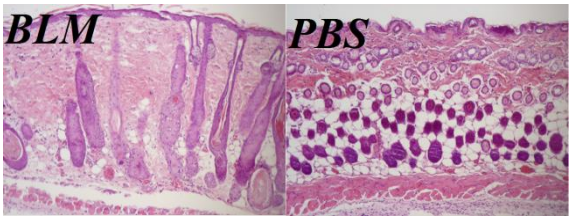

Figure 3: Skin histology on the 21st day, haematoxylin and eosin stain at a magnification of x 100. In BLM group: flatness of epidermis, thickened derma, homogenisation of collagen fibres, hyperplasia of hair follicles, dermal adipose layer depletion, inflammatory infiltrate under panniculus carnosus. In PBS group: the structure of the epidermis and dermis is unchanged, inflammatory infiltrate under panniculus carnosus is detected.

Examples of measured fluorescence spectrum from the injection area at λ_e = 365 nm on the 21st day of the experiment is shown in the Figure 4.

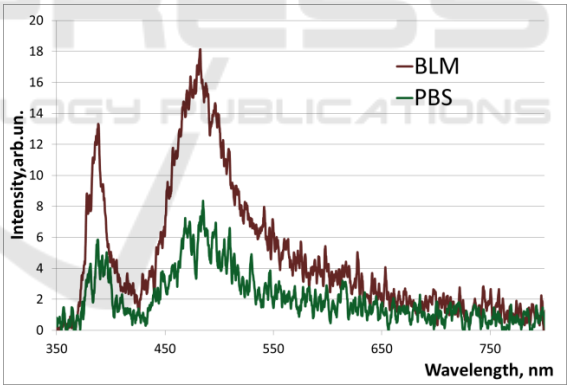

Figure 4: The example of the fluorescence spectrum in BLM and PSB groups on the 21st day of the experiment; λ_e = 365 nm.

Obtained spectra are characterized by the presence of two maxima corresponding to the backscattering peak (365 nm) and to the fluorescence of the collagen and elastin (455 nm).

Figure 5 illustrates the results of optical measurements and laboratory analysis.

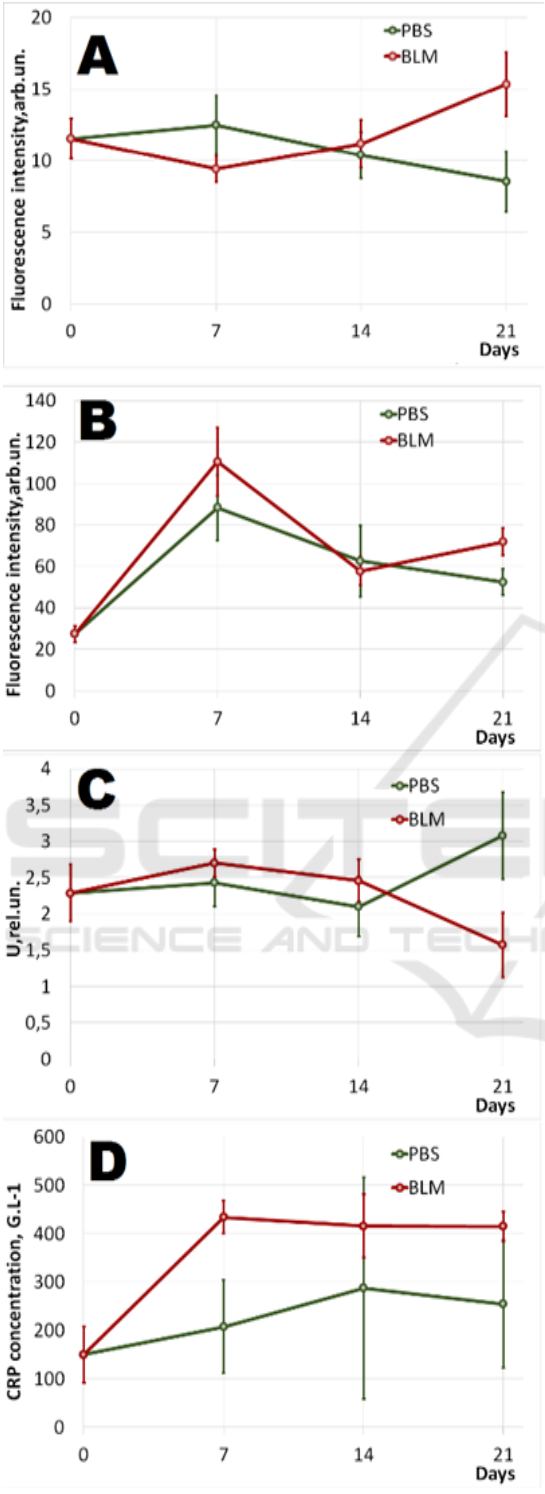

Figure 5: Dynamic by days: A. - Fluorescence intensity rate of collagen and elastin (λ_e = 365 nm, λ_f = 455 nm); B.- Fluorescence intensity rate of porphyrin (λ_e = 535 nm, λ_f = 610 nm); C. - Relative oxygen consumption rate in tissues; D. - C-reactive protein rates. 95% confidence interval is shown in the chart.

Figure 5A shows the dynamics of the t6yrteaverage fluorescence intensity rate of collagen and elastin in both groups. On the 21st day a statistically significant difference in BLM and PBS groups appeared.

The results of porphyrin fluorescence (Figure 5B) demonstrate the increase in the average fluorescence intensity as compared to the 0 day of the experiment in both animal groups. Besides that, a statistically significant difference in BLM and PBS groups were received on the 21st day.

The results of relative oxygen consumption rate demonstrate its statistically significant decrease by the 21st day in BLM group (Figure 5C).

Figure 5D shows the increase in C-reactive protein rate of animal blood serum in both groups. Statistically significant differences in BLM and PBS groups were assessed on the 7th day of experiment.

All data obtained during the experiment leads to a number of conclusions and assumptions. Thus, we believe, that the increase in endogenous fluorescence intensity rate of collagen and elastin on the 21st day in BLM group is due to its accumulation in the histologically confirmed fibrosis area. Whereas collagen is the main extracellular substance of connective tissue in skin fibrosis (Ho et al., 2014), we consider, that the impact of elastin fluorescence is imperceptible.

Tissues in which collagen fibers are extensively synthesized are known to have a high oxygen requirement. Nevertheless, the formed fibrosis decreases it due to the reduction in the number of cell elements (Lokmic et al., 2012). The relative oxygen consumption rate on the 21st day in BLM group is representative of this causation. (Figure 5A and 5C). Some researchers consider that fibrosis is permanent when a tissue becomes few-celled and has a lack of biologically active molecules essential to the extracellular substance deterioration (Iredale, 2007; Wynn, 2008, Rockey, 2015). We believe that contemporary examination of collagen fluorescence and the relative oxygen consumption rate in tissues will allow to establish the synchronous nature of this process. The information received is essential to clinicians as the data on the degree and the rate of skin fibrosis progression in systemic scleroderma enable to diagnose this form of disease, to determine a management strategy in time, and to predict the clinical course.

Porphyrins are well known to response on metabolic changes in tissues quickly. Their synthesis is particularly enhanced in cells during inflammation and hypoxia (Petritskaya et al., 2015). Vasculopathy is known to cause the oxygen delivery decrease in

cells at the beginning of scleroderma. In the following, fibrosis tissue induces perfusion defect and becomes the main cause of hypoxia (Van Hal et al, 2011). Since bleomycin-induced model better represents tissue fibrotic changes (Yamamoto et al, 2011), we suppose that statistically significant magnification in porphyrin fluorescence rate on the 21st day indicates a chronic hypoxia and is based on the perfusion defect.

We also assumed the development of inflammation of the dermis during fibrosis formation in experimental model. High levels of blood serum C-reactive protein in BLM group on the 7th day were determined in support of it. That implies the disease high activity and is a significant predictor of complications and premature mortality (Muangchan et al., 2012; Darby et al., 2016). Hence, daily subcutaneous injections also were associated with inflammation under panniculus carnosus in both animal groups. Probably, the increase in porphyrin fluorescence intensity in both groups was due to it. Nevertheless, to prove our assumption a separate study needs to be carried out providing with the method development that will make it possible to distinguish chronic hypoxia and inflammation of different sites.

4 CONCLUSIONS

The use of optical technologies in the experiment enabled to determine the increment in endogenous fluorescence intensity of collagen and the decrease in tissue oxygen intake in the fibrosis area. We also registered the increase in endogenous fluorescence intensity rate of the porphyrins as a potential chronic hypoxia and inflammatory marker.

It is important that all optical methods used in this study were non-invasive. Nevertheless we managed to obtain quantitative and impersonal information. Considering the fact that the animal scleroderma model is relevant, the data obtained can be reproduced in man.

The results of the experiment, of course, demonstrate the necessity of a research continuation in this direction, but already now it is possible to predict great opportunities for optical technologies in the diagnosis of skin fibrosis.

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