Application of laser fluorescence spectroscopy in non-invasive assessment of ultraviolet-induced skin immune response \textit{in vivo}

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Abstract. Acute ultraviolet (UV) -induced skin damage is associated with structural alterations, vasodilatation and inflammatory response. Leukocyte infiltration is one of the main features of inflammation and could be found in the area of UV injury. It was shown that porphyrins which have well-known autofluorescent properties play a role in the chemoattraction of immune cells to the area of local damage. This study examined the possibility of application of laser fluorescence spectroscopy (LFS) in the assessment of ultraviolet-induced immune response in ICR mice. Animals (N=25) were exposed by UVB light and LFS was conducted on the dorsal skin of each mice 0, 0.5, 3, 6 and 24 hours after UV irradiation. Moreover, in every time point we performed skin biopsy and histology. Using LFS, time-dependent dynamic changes in the fluorescence parameters of porphyrins were found. Mentioned indices were in a good agreement with histological findings. Statistically significant correlation was found between the severity of inflammatory infiltrate and the tissue content index ($\eta$) of porphyrins (Pearson correlation coefficient: $r = 0.912, p = 0.031$). Achieved results not only have fundamental value but could be further investigated and applied in clinical practice: e.g. to objectively predict individual immunologic reaction to UV-light.

1. Introduction
The long and repeated effect of ultraviolet (UV) radiation on the human body is associated with serious consequences in the long term - the development of malignant tumors, photoaging, immunosuppression. It is known that the earliest manifestation of UV-exposure is acute nonspecific skin damage, which is associated with structural, vascular and immunological changes in the epidermis and dermis, and is clinically manifested as UV-erythema [1]. The cascade of inflammatory reactions, mediator molecules involved in damage to the epidermis and vasodilatation, as well as the dynamics of structural changes in the skin induced by acute UV exposure, are currently poorly understood [2]. Existing methods for assessing UV-induced skin alterations (morphological, biochemical, molecular) are invasive and not sensitive enough to early changes, and processing the results requires additional time and resources [3].

A number of studies have shown that porphyrins, nitrogen-containing orange-red fluorescent pigments, accumulate in inflamed tissues after mechanical, chemical, and radiation damage [4; 5]. In...
experiments conducted by Schneckenburger and colleagues using the LFS method, the accumulation of porphyrins in the area of artificially-induced skin inflammation in Wistar rats was shown [6]. It is believed that the source of porphyrins in tissues can be free heme, the concentration of which increases sharply in tissues due to hemolysis or excessive damage to cells induced by internal or external (e.g., high dose UV- exposure) stimuli. Hypotheses are put forward that porphyrin molecules play a role of chemoattractants and engage immune cells to come the site of inflammation and contribute to the formation of infiltrate [7].

Fluorophores, such as porphyrins, which are responsible for tissue inflammation and immune infiltration can also be detected using laser fluorescence spectroscopy (LFS) in red and green spectrum range [8]. This approach does not require consumables/reagents, is non-invasive, aseptic and allow to rapidly obtain ready-made quantitative data characterizing the pathological process.

Thus, the use of LFS for assessing UV-induced changes in the skin inflammatory immune response is promising.

2. Materials and methods

2.1. Animals and ultraviolet irradiation

The present study was conducted on male ICR mice aged 6-8 weeks (N = 25) weighing 28-35 grams. The animals were kept under standard vivarium conditions at a temperature of 21 – 23 °C, humidity 50 - 65%, 14 hour daylight. They received balanced granular food that did not contain fluorophores and had free access to drinking water. The animal quarantine period was 10 days. 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).

Acute UVB-induced skin damage was initiated in all experimental animals. Previously, 48 hours before the study, fur was removed in the dorsal area (using veet depilatory cream). Ultraviolet irradiation was performed using a therapeutic source of UVB with a wavelength of 311 nm - Dr. Honle Dermalight 500-1 (manufactured by "Dr. Henle Medical Technician GmbH", Germany). The intensity of the UV was previously measured at each stage of the experiment using a TKA-PKM 12 spectroradiometer (UV meter). During the irradiation, all animals were anesthetized to avoid stress and movements.

The mice were divided into 5 subgroups of 5 animals each. The intact subgroup (control, N = 5) was not exposed to UVB radiation. In animals of the remaining subgroups (except the 1st), an acute UVB damage to the dorsal skin was initiated. The distance to the source was 10 cm, the exposure time was 16 minutes. Eventually, 24 hours after irradiation, the skin of the mice was visually examined and photographed.

2.2. Optical measurements

Before irradiation and after 0.5, 3, 6 and 24 hours after we carried out LFS of the affected and intact skin of mice using LAKK-M system (SPE 'LAZMA' Ltd, Russia). The principal scheme of the device is described in the study by Chursinova et al. [9]. The process of registering optical parameters is presented in Figure 1.
Figure 1. The process of optical measurements

The “Fluorescence” operating mode of LAKK-M system implements the method of LFS. Using a fiber optic probe, low-power radiation from a selected source is delivered to the surface of the biological tissue. The output power at the distal end of the fiber-optical probe was about 2 – 3 mW for each light source. Secondary radiation is delivered to the spectrometer through the receiving fiber, and its spectrum is reflected on the monitor in real time.

To excite fluorescence in various parts of the visible and near UV spectra, source with wavelength $\lambda_e = 535$ nm was used. The wavelengths on which the fluorescence had a maximum value were marked with $\lambda_f$. Porphyrin is characterized by a two-hump fluorescence spectrum with maxima at wavelengths of 630 and 710 nm. In the wavelength range of 650 - 750 nm porphyrins make the main contribution to the endogenous fluorescence of biological tissue, but at a wavelength of 630 nm fluorescence of porphyrins is more pronounced. Although, other fluorophores (e.g., lipofuscin) may also fluoresce at this wavelength. Thus, for porphyrins the fluorescence wavelength $\lambda_f = 610-630$ nm was used.

To quantify fluorescence, the tissue content index of the fluorophore was used, which is calculated by the formula:

$$\eta_f = \frac{I_f \cdot \beta}{I_f \cdot \beta + I_{bs}}$$

where $I_f$ is the intensity at the fluorescence wavelength of a particular fluorophore, $I_e$ is the intensity at the wavelength of the laser used to excite fluorescence, $\beta$ is the attenuation coefficient of the used filter, $\beta \approx 1000$.

2.3. Histology

Laboratory animals were sacrificed before, 0.5, 3, 6, and 24 hours after irradiation and biopsy of the dorsal skin (skin flap 1.0 cm$^2$), material sampling and histological examination (using hematoxylin and eosin (H&E) strain) were performed. In addition, material sampling and pathomorphological examination were performed in intact mice. For each histological sample, the number of inflammatory cells (polymorphonuclear cells) was counted in 10 high-power fields (hpf). Further, the immune infiltrate was graded as pronounced (400-600 cells), moderate (200-400 cells), weak (less than 200 cells).

3. Results and discussion
When analyzing the results of LFS, dynamic changes in the fluorescence parameters of porphyrins over time were noted. The results of optical measurements are presented in Figure 2.

![Fluorescence Spectrum](image)

**Figure 2.** Dynamic changes in tissue content index of porphyrins, $\lambda_e=535$ nm

Peak values of the tissue content index ($\eta$) of porphyrins were found after 3 hours after UV exposure. Then a gradual decrease in $\eta$ of porphyrins was observed - after 24 hours its values closely approached normal levels (as in unirradiated skin). An example of fluorescence spectra after 3 hours is presented in Figure 3.

![Fluorescence Spectrum Comparison](image)

**Figure 3.** The example of fluorescent spectrum: intact (without UV-exposure, 0 hours) and irradiated skin (3 hours after UV-exposure), $\lambda_e=535$ nm

During the analysis of pathomorphological samples, we were able to detect dynamic structural, vascular and inflammatory changes at the different time points after UV exposure. So, 30 minutes after irradiation, the first signs of an acute inflammatory response were noted: vasodilation, an influx of neutrophilic leucocytes. At the point of 3 hours, the inflammatory response reached its peak: abundant neutrophilic infiltration developed, and after 6 hours the immune infiltrate became less pronounced and had mixed structure (neutrophils and histiocytes) and single «sunburn cells» were noted. After 24 hours, the stabilization of the inflammatory reaction was observed: lymphocytic-histiocytic infiltrate was widely distributed in the skin, specific signs of UV damage appeared («sunburn cells», vacuolization of the basal cell layer) and were also uniformly distributed in the thickness of the epidermis.
Figures 4 and 5 show the histological changes that occurred 3 hours after UV exposure compared with unirradiated skin (hematoxylin and eosin staining).

**Figure 4.** Histology of unirradiated skin: the structure of the epidermis and dermis is unchanged, there no signs of inflammatory infiltrate (H&E stain, magnification of x 400).

**Figure 5.** Histology of skin 3 hours after UV-irradiation: acute inflammation with marked infiltration of neutrophils (H&E stain, magnification of x 400).

Using Pearson correlation coefficient, we found a statistically significant relationship between the severity of inflammatory immune infiltrate and the tissue content index (η) of porphyrins (r = 0.912, p = 0.031).

The obtained data had pathogenetic basis: the porphyrins, that are released from damaged and destructed skin cells and accumulated in area of UV-irradiated skin, play a role chemoattractants for neutrophilic granulocytes. It was found that the peak of neutrophilic infiltration 3 hours after UV exposure coincided with the peak porphyrins fluorescence. In addition, a marked increase in porphyrins fluorescence may be associated with the pronounced vasodilation observed in the UV-injured skin.

4. Conclusion

In this work, the applicability of LFS in non-invasive quantification of UV-induced changes in the skin immune response was demonstrated. The results are useful in terms of obtaining new fundamental data and could be applied in clinical practice: e.g. to objectively predict individual immunologic reaction to UV-light and to assess the minimal erythema dose.

Acknowledgments

The publication has been prepared with the support of Moscow Regional Research and Clinical Institute "MONIKI" within the framework of financing under the section "Science".

The reported study was funded by «UMNIK» grant provided by Innovation Promotion Fund (project «Development of a method for determining the minimal erythema dose», 2019).

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