In Vivo Experimental Detection of Inflammatory Process in Tissues by Fluorescence Spectroscopy

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Abstract: Laser fluorescence spectroscopy (LFS) is widely used in medicine. Today, oncology and oncosurgery are considered as the most promising fields of its application. It is known that cancerous tissues are able to accumulate different porphyrins, both endogenous and exogenous, in enhanced amount due to increased metabolism in cancerous cells. So, LFS can be used *in vivo* for detection of malignant tumours as well as for real-time intraoperative imaging or diagnostics at a photodynamic therapy. One of the reason of the enhanced accumulation of porphyrins in tissues is a chronic hypoxia. Therefore, it was hypothesized, that LFS could also be used for diagnosis of local inflammation in tissues. Recently, some indirect data confirming the hypothesis was obtained when observed inflammation due to invasion of external substances into tissues. This study proves the hypothesis in a direct experiment with animals and laboratory tests. Enhanced fluorescence intensity of the exogenous photosensitizer was found in inflamed tissues. The direct association between intensity of the fluorescence, histological characteristics and blood test results was shown. It was found that the registered fluorescence signal correlates with neutrophil counts in blood of tested animals. It proves that LFS could be an effective tool for registration of local inflammation.

1 INTRODUCTION

Laser fluorescence spectroscopy (LFS) is currently one of the promising methods for a non-invasive (*in vivo*) characterisation of biological tissues and its functional conditions (Johansson et al., 2008), (Tuchin, 2002), (Mycek et al., 2003), (Rogatkin et al., 2013). LFS is based on a registration of fluorescence spectra and (or) fluorescence intensities of endogenous or exogenous fluorophores on a surface of the tested tissues. A great interest of researchers to the method can be explained by its advantages over other methods of assessment of soft biological tissues. LFS differs from other methods by noninvasive modality (minimally invasive, if special preparations are used). It gives a possibility of *in vivo* diagnosis in real time, and is safe for the body (the method implies low-power laser light).

Among others areas, oncology and oncosurgery are considered as the most promising fields of application of noninvasive LFS in medicine of today (Tuchin, 2002). It is known, that cancerous tissues accumulate different porphyrins and its derivations, both endogenous and exogenous, in enhanced amount due to the increased metabolism in malignant cells (Mycek et al., 2003). Therefore, LFS can be used *in vivo* for a detection of malignant tumors as well as for real-time intraoperative imaging in oncosurgery or for a diagnostics at a photodynamic therapy. A great number of medical publications deal with the application of LFS for early detection of malignancies in skin (Calin, et al 2013), oral mucosa (De Veld et al., 2005), gastrointestinal tract (Duraipandian et al., 2012), (Koizumi et al., 2013), and urogenital system (Stenzl et al., 2011), (Karaoglu et al., 2014), as well as for cancer diagnostics at a photodynamic therapy (Andersson-Engels et al., 1995). In oncology LFS can help surgeons to visually distinguish healthy tissue from the cancerous one and to perform a precise ablative process (Vahrmeijer et al., 2013). Also, LFS can be used to identify sentinel lymphatic nodes by providing their realtime intraoperative imaging. Furthermore, it can be used to prevent iatrogenic damage to vital structures, such as the ureter or nerves (Handgraaf, 2014). There are many

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publications on the use of this method for intraoperative imaging in the breast surgery, gynecology, neurology and other medical specialties (Handgraaf, 2014), (Tummers, 2014), (Sugie, 2013). Application of LFS seems also to be very promising in the robotic assisted surgery (Hellan,2014). All these application of LFS are based on the fact of the abnormal fluorescence of both endogenous and exogenous fluorophores in tumorous tissues.

Meanwhile, it was shown previously, that one of the reason of the enhanced accumulation of porphyrins in tissues is a chronic hypoxia (Rogatkin et al., 2009). Therefore, it can be hypothesized, that LFS could also be used for diagnosis of a local inflammation in tissues. Currently, the search of noninvasive methods of local inflammation diagnosis is an extremely actual task, especially in the regenerative medicine, when local regenerative processes are under investigations and control. Also, it is important in oncology. It is well-known that a lot of pathological changes in tumorous tissues, in particular in cancerous ones, are accompanied by a number of local and systemic inflammatory responses (Diakos, 2014). What if the enhanced accumulation of porphyrins in a tumour is caused not only by malignat processes, but also by inflammatory ones, and it is not specific for cancerous cells? Today, there is a few data only devoted to the differentiation of the fluorescence spectra for a cancer and inflammation. So, this issue requires further attention (Lv, 2015), (Zhang, 2013), because of potential errors can exist in LFS due to this phenomenon.

Generally, in clinical practice of today, a leukogram response test is widely used for the inflammation diagnosis. Leukocytosis, neutrophilia, left shift of the leukogram, etc. are the frequent blood hallmarks of inflammations (Marshall, 2006). However, the leukogram is nonspecific indicator of local inflammations. It is not able to specify the location of the inflammatory process. Biopsy and subsequent histological examination can accurate and reliable detect of any local inflammations, but it is an invasive method. There are publications on the use of thermography as a noninvasive quantitative imaging method for assessing the local inflammation (Christensen, 2014), (Arfaoui, 2012). However, the increase in temperature is only one of signs of local inflammation and, therefore, may be considered as an auxiliary method. The rise in temperature is not pathognomonic for local inflammation and may indicate a normal physiological reactions (enhanced functional activity of muscles, heating of skin, etc.). Another modern method for local inflammation

diagnosis is a scintigraphy (Love, 2013). However, this method involves an introduction into the organism of radioactive isotopes, so it is associated with dangerous ionizing radiation exposure. Thus, the search for new, noninvasive or minimally invasive, and real-time instrumental methods for diagnosis of a local inflammation is an extremely important task.

Recently, some indirect data confirming the hypothesis of enhanced accumulation of exogenous fluorophores of an aluminum phthalocyanine series in inflammed tissues were published (Petritskaya et al., 2014). Enhanced fluorescence was observed at local inflammation due to invasion of external substances into tissues. But in the reffered study the histological or a blood test confirmation of the inflammation process wasn't been done. The aim of our current study was to confirm possibilities of LFS to detect *in vivo* a local inflammation in tissues in a direct experiment with animals and laboratory tests.

2 MATERIALS AND METHODS

The study was performed in white laboratory mice (N=12) and was conducted in accordance with all ethical principles formulated in the Declaration of Helsinki on the care and use of animals in research and the Regulations of the European Science Association (86/609/EC).

Local inflammation was provoked as follows. All procedures were carried out under general anesthesia (Zoletil + Xylazine). An incision was made in the lateral part of the inguinal fold, then skin was separated from the fascia by blunt dissection, and the underlying muscle was clamped at a distance of 7 mm from the incision by a Mosquito clamp. The size of the resulting zone of injury was 3x3x3 mm (Figure 1). Then, the photosensitizer "Photosens" based on aluminum phthalocyanine was injected intraperitoneally in the dose of 2 mg/kg. It is known, that the fluorescent signal of this photosensitizer in tissues can be detected during 4-5 weeks, so it is possible to use the aluminum phthalocyanine based photosensitizer in prolonged experiments without any additional injections. To prevent side effects, experimental animals were not exposed to direct sun radiation during the experiment, so any phototoxic effects did not affect the laboratory mice.
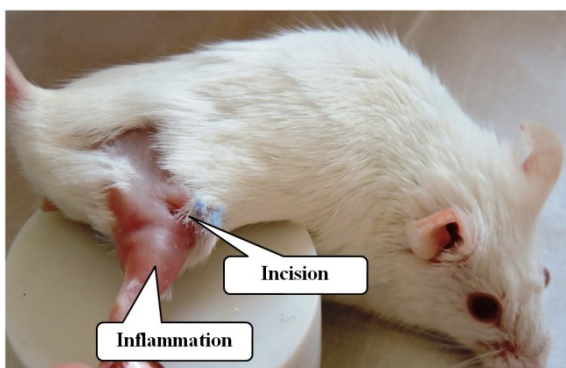
Figure 1: Position of the inflamed region relative to the incision.

To confirm the fact of inflammation, hematology tests as well as a conventional histological analysis of the affected area of the hind limb of mice were done prior the provocation and on Days 3 and 10 after the provocation of the local inflammation. For this purpose, two mice every time points (Days 0, 3, 10) were withdrawn from the experiment. Since our interest was only to confirm the presence of local inflammation without assessment of any specialties of the inflammation, it was sufficient to carry out both histological examination and a blood test only in these three points of time (Days 0, 3, 10) as the most expressive points of the evolution of such inflammation. In hematology test results we firstly took into account the percentage ratio of neutrophils (neutrophil counts) as the most informative parameter at a local inflammation in animals.
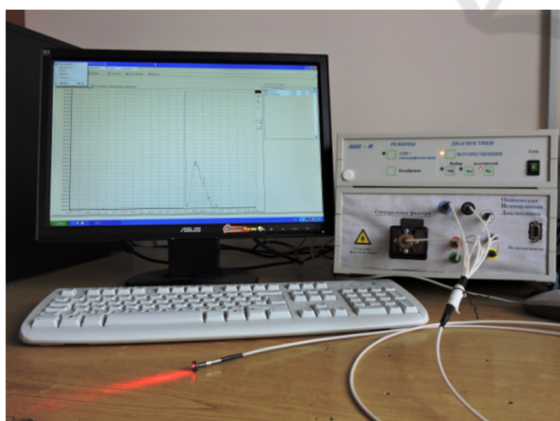

Figure 2: The diagnostic system "LAKK-M".

In all experiments, fluorescence was recorded *in vivo* with the use of laser diagnostic system LAKK-M in the "Fluorescence" operation regime. The system is equipped with fiber optical probe (Figure 2). Excitation of tissue fluorescence was made in the

continuous wave (CW) mode at the wavelength 635 nm (a semi-conductor laser). Power of the laser radiation on a distal end of the optical fiber probe (on a surface of tissues) was around 5 mW. Fluorescence intensity was measured at 670 nm – in a maximum of the fluorescent spectrum of the used photosensitizer "Photosens". Subsequently, the intensity at this wavelength will be called "*fluorescence intensity*".

Measurements of the fluorescence intensity in animals were carry out before the provocation of the local inflammation (before the injection of the photosensitizer and on Days 2, 3, 6, 8, 10 and 16 after the provocation and the injection.

Examples of fluorescence spectra from inflamed tissues and from a contralateral region are shown in Figure 3.
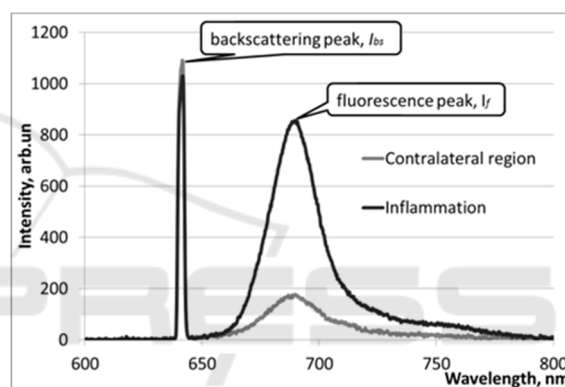

Figure 3: Examples of fluorescence spectra from the contralateral region and from inflamed tissues (3 days after the injection of the photosensitizer).

To study the dynamics of photosensitizer accumulation in inflamed tissues, the peak value of the measured fluorescence intensities was tracked in time. Also, to clarify the influence of the initial laser radiation power as well as of local optical properties of tissue on the registered intensities, we compared the dynamics of the measured fluorescence intensities and the dynamics of the coefficient of fluorescence contrast K_f (Rogatkin, et al 2013), which calculated using the intensities as follows:

$$K_f = 1 + \frac{I_f \cdot \beta - I_{bs}}{I_f \cdot \beta + I_{bs}}$$

where K_f is the coefficient of fluorescence contrast ($0 < K_f < 2$); I_f is the maximum of the fluorescence intensity; I_{bs} is the measured intensity of the backscattered radiation at the excitation wavelength; β is an instrumental reducing coefficient ($\beta \approx 1000$ to diminish I_{bs} to a level which is comparable with the level of I_f). In this coefficient I_f is normalized both

by local optical properties of tissues and by a power of excitation radiation, so it is less sensitive to theirs changes.

To compare the fluorescence intensity from inflammation and contralateral areas, an index of inflammation intensity $\mu(\lambda_f)$ was calculated as follows:

$$\mu(\lambda_f) = \frac{I_f(\lambda_f)}{I_{f0}(\lambda_f)}$$

where I_f is the fluorescence intensity from the inflamed area, I_{f0} is the fluorescence intensity from the contralateral region, and λ_f is the fluorescence wavelength (in our case $\lambda_f = 690$ nm).

3 RESULTS AND DISCUSSION

All experimental results showed an enhanced intensity of the fluorescence signal in the injured tissues, compared to that in the contralateral region. Figure 4 presents the averaged data for the group of mice.
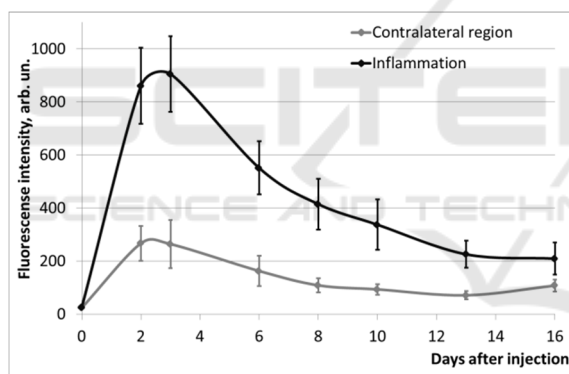

Figure 4: Dynamics of fluorescence intensities.

Earlier it was shown (Rogatkin et al., 1998), that uncertainty of results of such measurements in the laser fluorescence diagnostics amounts to 40% of the measured value. Therefore, the experimentally observed differences between signals from the intact area and the inflamed tissues are significant. This suggests that inflammatory processes can be *in vivo* detected by LFS.

The dynamics of K_f is shown in Figure 5. It is easy to see that there are no fundamental differences in the behavior of the curves in Figures 4 and 5. It confirms, that the influence of the laser power fluctuation or an influence of local optical properties of tissues on registered intensities is small enough at so high fluorescence of the photosensitizer used.
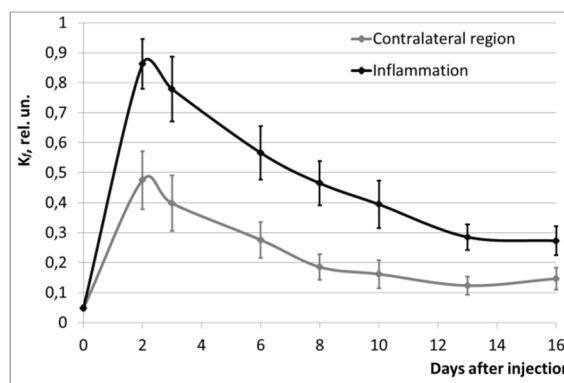

Figure 5: Dynamics of the coefficient of fluorescence contrast.

Dynamics of the index of inflammation intensity $\mu(\lambda_f)$ versus days is shown in Figure 6. For clarity, the time points of hematology and histology tests are denoted by crosses.
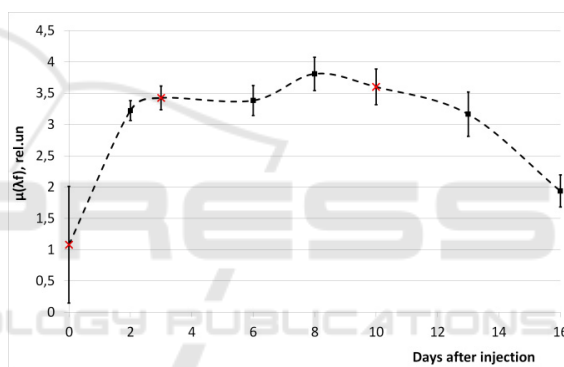

Figure 6: Dynamics of the inflammation intensity index $\mu(\lambda_f)$.

All data of laboratory blood tests showed an increase in the neutrophil counts (Figure 7) and a relative increase in the band neutrophil counts (Figure 8), i.e. a shift of the leukogram to the left. These parameters indicate exactly the occurrence of inflammatory process in organism.

Histological examination performed at Day 0 before the provocation of inflammation was normal. Histological examination performed at Day 3 after the provocation revealed an acute inflammatory response in the injured muscle tissue, dermis and subcutaneous adipose tissue, namely: there were edema, leukocyte inflammatory infiltration of tissues, and necrotic foci in muscle. Reduction of inflammatory activity and some regenerative muscle tissue changes were observed at Day 10. But the inflammatory infiltrate, indicating a continuing inflammatory process, remained in the dermis and subcutaneous adipose tissue.
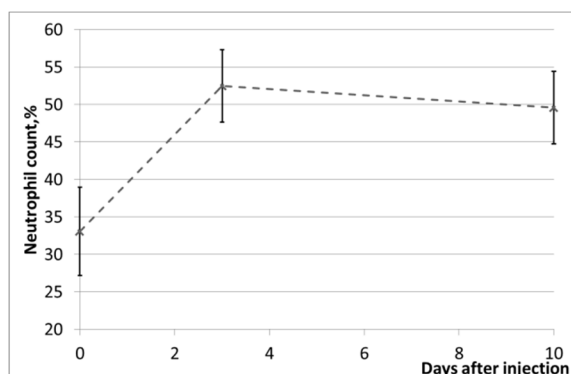
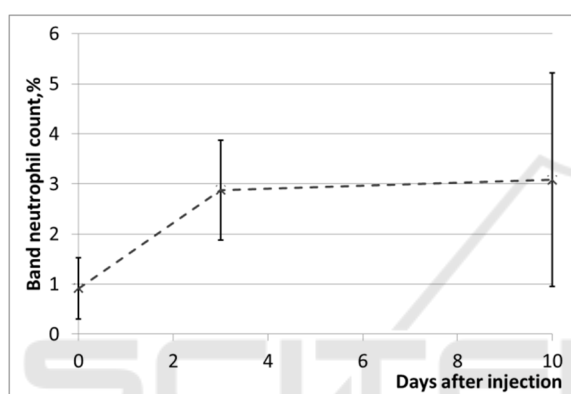Figure 7: Changes of blood neutrophil counts in laboratory mice.

Figure 8: Changes of blood band neutrophil counts in laboratory mice.

Based on these results (Figures 7 and 8), it can be assumed that the inflammation intensity index $\mu(\lambda_f)$ in inflammation regions correlates well with blood neutrophil counts. The increase of the index $\mu(\lambda_f)$ on Day 3 corresponds to an acute local inflammatory response, which was evident in both hematology tests and at histological assessment. On Day 10, the $\mu(\lambda_f)$ remained high, correlating with histologically confirmed persistence of the inflammation in subcutaneous adipose tissue and dermis, and with the increase in the blood neutrophil counts.

4 CONCLUSIONS

The aim of the current study was to confirm possibilities of LFS to detect *in vivo* a local inflammation in tissues. In a direct experiment with animals and confirmational laboratory tests it was shown and proved that non-malignant inflamed tissues can have an enhanced accumualtion of exogenious photosensitizer and, therefore, can have an enhanced superficial fluorescence of it like several cancerous tissues have. Furthermore, it was shown that the intensity of the fluorescent signal in the inflamed tissues correlated with blood neutrophil counts and was associated with changes in histology.

It is of special note, that this result is very important both for an experimental research and for a practical medicine. Many processes in a human body are associated with development of local inflammations. First of all, these are different processes related to mechanical and thermal tissue damage, to introduction of foreign agents into a tissue, at a transplantation, for example. So, a diagnosis of a local inflammation by LFS technique *in vivo* can be used as a navigation method in surgery and, also, as an intraoperative assessment of tissue conditions at a regenerative surgery. These data is important for the fluorescence diagnostics in oncology, as well. It was shown that the exogenous photosensitizer can be accumulated not only in malignant cells, but also in the area of inflammation. It makes us reassess the applicability limits of LFS at a photodynamic therapy.

Radiation therapy is an effective and accepted treatment modality in oncology. Various radiation-induced reactions, including inflammation, could be its side effects, which is the reasons why radiation doses are often fractionated. However, fractionation procedures are poorly individualized, and standard radiation regimens are used due to a lack of affordable instrumental method for assessment of individual local inflammations. The proposed technique could become such a method. So, in a view of the foregoing, it can be assumed that this method of diagnostics of a local inflammation may have a broad clinical application.

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