In vivo fluorescence diagnostics and photodynamic therapy of gastrointestinal superficial polyps with aminolevulinic acid. A clinical and spectroscopic study*

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In the present study initial results of clinical study related to the treatment of patients having different types of precancerous lesions in the area of esophagus, stomach and intestine by photodynamic therapy (PDT) based on aminolevulinic acid (ALA-PDT) are reported. The procedure was performed by laser fibre system with the light guides introduced through biopsy channel of an endoscope. In addition, *in vivo* fluorescent diagnostics and spectral analyses of biopsies were performed. Each patient had a positive response to therapy. In two cases there was a total response and in other five cases more than sixty percent of suspected area was removed. Additionally, sigilocellular carcinoma of stomach was revealed in one case. It appears from the results of this study, that the treatment of precancerous lesions with ALA-PDT could be successful treatment modality.

Key words: fluorescence diagnostics, photodynamic therapy, protoporphyrin IX

Photodynamic therapy (PDT) is a minimally invasive therapeutic modality approved for the treatment of neoplastic and vascular diseases [1]. It involves light-induced activation of administered photosensitizer in tissue to produce local necrosis or apoptosis thus it is performable only in the place where light can be delivered [2]. Nowadays there are three clinically approved agents used in PDT and one of them is aminolevulinic acid (ALA). ALA is not the photosensitizer but it is a metabolic precursor of light sensitive product protoporphyrin IX (PpIX) in heme biosynthetic pathway [1]. Selective accumulation of PpIX in malignant tissue after administration of ALA has been reported [2, 3]. Although ALA-PDT is clinically approved mainly for the treatment of actinic keratosis and basal-cell carcinoma of the skin it could be also used for intra corporal malignancies [1]. However, it was shown, that ALA-PDT is not suitable for treatment of thick lesions (more than several milimetres) [6, 12]. There are several advantages of application of ALA. The time interval between drug administration and illumination of the lesion is rather short, namely 4 hours as compare with other photosensitizers, where this interval is from 24 to 96 hours [1, 5]. The clearance of PpIX after drug administration does not exceed 48 hours [4]. With using others photosensitizers the clearance is in interval from several weeks to several months.

Upon irradiation of photosensitizer energy of absorbed photons is partially transferred to molecular oxygen through a metastable triplet state and partially spent on fluorescence. Fluorescence is a useful tool for detection of accumulation of a photosensitizer in treated tissue what can be utilized in fluorescence diagnostics. The field of application of fluorescence diagnostics has been expanded after development of laser techniques. With fiber laser systems it is now possible to deliver light to and subsequently collect radiation from the places, which are reachable only with difficulties by common light sources like e.g. esophagus, intestine.

A valuable complement of *in vivo* fluorescence diagnostics is fluorescence investigation of biopsies. The fluorescence image of a cryoslit from biological sample with accumulated photosensitizer is a mixture of signals due to autofluore-

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scence (originating from NADH, flavins, flavoproteins etc) and a signal from a photosensitizer [9]. Such signals are possible to distinguish by using methods of spectrally resolved fluorescence microscopy and linear unmixing. This enables monitoring of the level of accumulation of photosensitizer in suspected area of tissue giving valuable information for improvement of the whole PDT procedure.

In the present study, we have applied ALA-based PDT for the treatment of superficial precancerous lesions in the area of esophagus, stomach and intestine. *In vivo* fluorescence detection of photosensitizer accumulation in the tissue was performed by means of a novel fiber spectrometer. The results of *in vivo* fluorescence diagnostics were compared with fluorescence analysis of biopsies.

Patients and methods

Eight patients (three female, mean age of 63), four with superficial esophageal lesions with fragile surface one with sigilocellular carcinoma of stomach and three with superficial intestinal dysplastic adenomas were involved in the present study. After an informed consent had been obtained, patients were given 40 mg/kg ALA dissolved in 10 ml of fruit juice to drink. They were asked to wear sun-glasses and stay in the dark room in order to avoid exposure to sunlight. Four hours later endoscopic treatment was performed. For detection of accumulated PpIX in tissue, fibre spectrometer LESA-01-BIOSPEC (JSC BioSpec) with He:Ne laser operating at 632.8 nm with power 2 mW was used (Fig. 1). The output end of spectrometer fiber consist of a central light-guide, delivering the light to the specimen and ambient light-guides, collecting reflected and emitted light from the specimen. Fluorescence emission spectra recorded from both suspected area and adjacent normal mucosa were compared before PDT. Those of the treated tissue were measured shortly after the PDT treatment as well as one week later during the endoscopic control.

Biopsies were taken and immediately frozen in liquid nitrogen before PDT treatment. Cryoslits prepared from the biopsies were scanned by laser scanning confocal microscope LSM 510 Meta (Zeiss) with C-Apochromat 40x/1.2 water immersion objective. The samples were excited by 458 nm Ar:ion and 633 He:Ne laser lines (Lasos Lasertechnik), respectively. Emission was detected with spectral META detector at 22 different channels quasi-simultaneously. Linear unmixing methods were used to determine the regions of PpIX accumulation.

For PDT procedure diode laser system LPhT-630/675-01-BIOSPEC (JSC Biospec) emitting light with wavelength of 632 nm was used. The light with power of 400 mW was delivered via fibre with 4 cm long diffusing end introduced through biopsy channel of endoscope interstitially for 120 sec., giving the total dose of 48 J/cm^2.

Results

In vivo fluorescence measurements. *In vivo* fluorescence measurements performed 4 hours after ALA administration before PDT revealed increased fluorescence intensity in the suspicious area of the tissue as compared to the adjacent normal mucosa (Fig. 2).

Fluorescence emission spectra recorded from the suspicious area and the adjacent normal mucosa after the excitation with 632.8 nm light were characterized by similar shape with a maximum at around 698 nm but different emission intensity. Significant decrease of fluorescence intensity

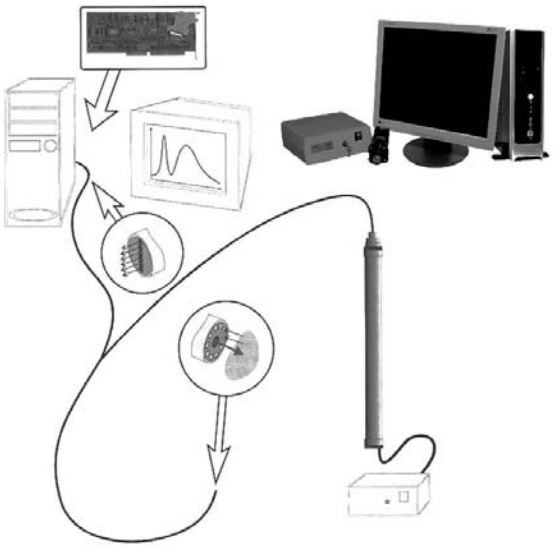

Figure 1. Scheme and photography of LESA-01-BIOSPEC.

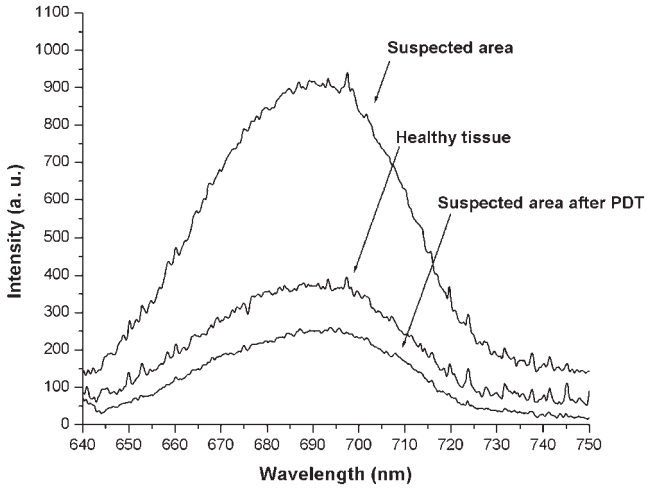

Figure 2. Fluorescence spectra from suspicious area, healthy tissue and from suspicious area after PDT treatment. Excitation was 632.8 nm.

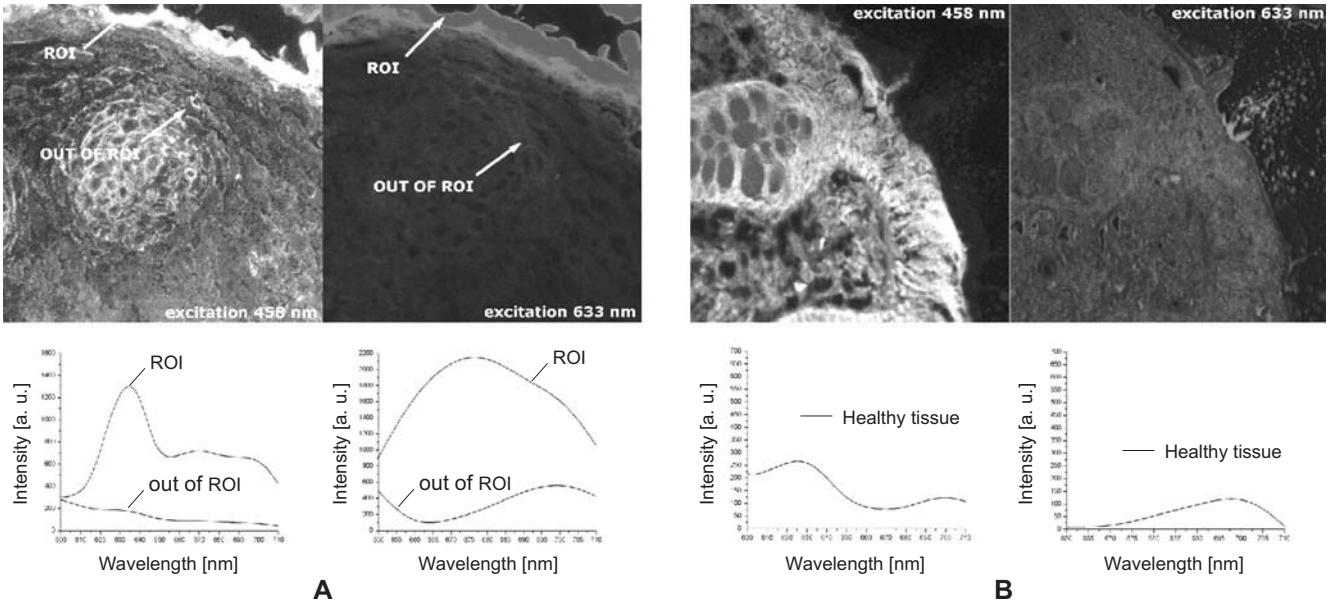

Figure 3. Spectral images of cryoslits from (A) suspicious area of oesophageal tissue and (B) healthy mucosa before PDT. Fluorescence spectra after excitation at 458 nm (left) and 633 nm (right) corresponding to the areas with (ROI) or without (out of ROI) visible red fluorescence are shown below the images.

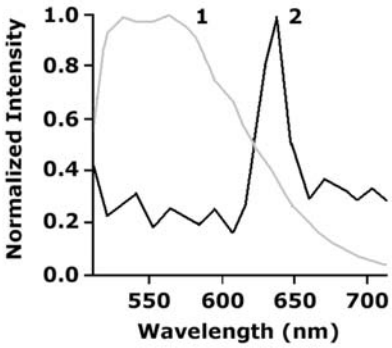

Figure 4. Reference spectra of flavin autofluorescence components (NADH + FAD) (1), Protoporphyrin IX (2).

wasfound for treated area immediately after PDT procedure. At the time of endoscopic control – one week after PDT, the fluorescence intensity recorded from the treated area was comparable to the normal mucosa.

Fluorescence microscopy. Examples of spectral images of cryoslits from biopsies of suspicipious area from a sample of superficial esophageal polyps and from healthy tissue are shown in Figure 3. Areas of increased red fluorescence were clearly visible within the suspicious area (Fig. 3A). Corresponding fluorescence spectra were characterized by maxima at around 633 nm and 675 nm after the excitation with 458 and 633 nm light, respectively. In addition, an emission band at around 703 nm could be resolved in the spectra. Emission was found to be more intensive in the suspicious area than in the healthy tissue. For the healthy tissue maxima around 633 and 703 nm were recorded.

Linear unmixing of spectral images from cryoslits was performed using reference spectra of Protoporphyrin IX, Flavine Adenine Dinucleotide reduced form (FAD) and Nicotinamide Adenine Dinucleotide reduced form (NADH). Fluorescence emission spectra of a mixture of FAD and NADH in physiological solution and of PpIX in phospholipids membranes after the excitation with 458 nm are shown in Figure 4. The differences in these emission spectra allowed us to use linear unmixing method for microscopic spectral images and separate fluorescence signal due to PpIX from autofluorescence (Fig. 5). Resulting spectral images showed that the red fluorescence resulted merely from accumulated PpIX.

PDT. PDT performed 4 hours after ALA administration with a red laser giving the total light dose of 48 J/cm^2 resulted in the apparent reduction of superficial precancerous lesions. Each patient had a positive response to therapy. In two cases there was a total response and in other five cases more than sixty percent of suspicious area has disappeared at the time of endoscopic control (i.e. one week after PDT treatment). In most of the cases endoscopy showed flattening of the polyps (Fig. 6) and appearance of a white necrotic center at the site of the treatment. The surrounding mucosa appeared normal without grossly discernible changes after PDT. In one case a sigilocellular carcinoma of stomach was additionally diagnosed. Side effects, namely sunburn skin photosensitivity in three cases and mild pain in epigastrium in one case were registered (Tab. 1). In the rest of the cases, patients tolerated the ALA dose and the therapy without notable problems.

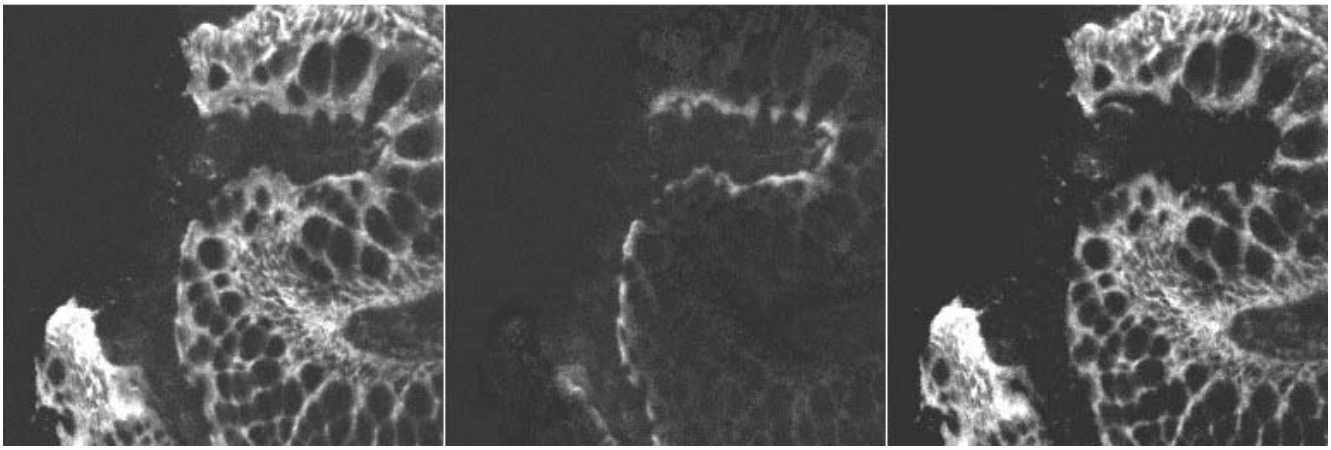

Figure 5. Linear unmixing of spectroscopic images from suspected area of tissue. Excitation was 458 nm. Initial spectral image (left), only PpIX fluorescence areas (middle), FAD/NADH fluorescence areas (right).

Discussion

In the present study ALA-PDT as a procedure for the treatment of superficial precancerous lesions in the area of esophagus, stomach and intestine was presented. Initial results of our clinical study confirmed that although ALA is clinically approved for the treatment of actinic keratosis and basal-cell carcinoma of the skin it can be used also for intra corporal malignancies [1]. Photosensitization with ALA turned out to be a feasible mode for the treatment of precancerous polyps and superficial lesions of esophagus, stomach and intestine, consistently with other results in this field [6, 13, 15–17]. While our treatment procedure resulted in total response in 25% of the cases, in a similar study related to the treatment of oncological patients with esophageal cancer by PDT with polyhaematoporphyrin only partial improvement but not total response could be obtained. Underlying reason is probably due to lower tumor selectivity of polyhaematoporphyrin [8]. On the other hand in a study related to the treatment of Barrett's low-grade dysplasia by ALA-PDT post-treatment biopsies showed no dysplasia in 98% of patients [15, 16]. Also the treatment of adenocarcinoma in Barrett's esophagus resulted in elimination of cancer in 77% of 22 patients [15]. Other study showed that severe dysplasia and superficial (≤2 mm) mucosal carcinoma of the esophagus can be ablated completely by 5-ALA-PDT with using 60 mg/kg of ALA and total dose 150 J/cm^2 [17].

Our results indicate that tumor necrosis can be accomplished without significant gross damage to surrounding normal mucosa. In general, toxic reaction of ALA is low and involves three areas. Sunburn is a hazard if the patient is not kept in subdued light for approximately 48 hours [6, 14]. Relatively mild elevations in liver function test results may also occur, but these

Table 1. Summary of ALA-based PDT for the treatment of superficial precancerous lesions of oesophagus and intestine

Oesophagus-Stomach					
P. No.	Age	Sex	Diagnosis	Result	Side effect
1	51	M	polypoid superficial lesion with fragile surface in oesophagus	70% reduction of original lesion, presence two small polyps (1 – 2 mm)	none
2	68	F	several superficial polypoid lesion (20 x 20 mm) in stomach	partial regresion of lesion with moderate necrosis. Histology: Sigilocellular carcinoma of stomach	photosensitivity of the face and hands for two days
3	61	M	local adenomatosis of stomach mucosa	macroscopic total response, without polypoid or ulcerous lesions	none
4	74	M	superficial adenoma of stomach (20x20 mm)	approximately 66% destruction with fllatening bottom	none
5	75	F	reduced residue of hypertrophic crease in stomach	macroscopic total response, without ulcerous lesions	mild pain in epigastrium for three days
Intestine					
P. No.	Age	Sex	Diagnosis	Result	Side effect
1	53	M	residue of adenoma polyps, after 4 APC treatments	total flattening, presence of 4 small (2 mm) ulcerations, without fluorescence	photosensitivity of the face, mouth and hands for three days
2	63	F	superficial adenoma polyp of rectum with dysplasias	flattening basis of original polyp	mild photosensitivity on dorsal side of hands for four days
3	61	M	superficial adenoma with dysplastic changes	small residue of original adenoma (1x 1mm), 60% reduction of original adenoma	none

are transient [4]. Finally, mild nausea and occasional vomiting may occur shortly after ingestion [4, 6, 13]. Consistently, only mild side effects were noticed in our study. Skin photosensitivity reported in 3 cases lasted no longer than 3 days contrary to several weeks typical for the most commonly used photosensitizer Photofrin [1, 12]. Mild pain in epigastrium occurred only in one case.

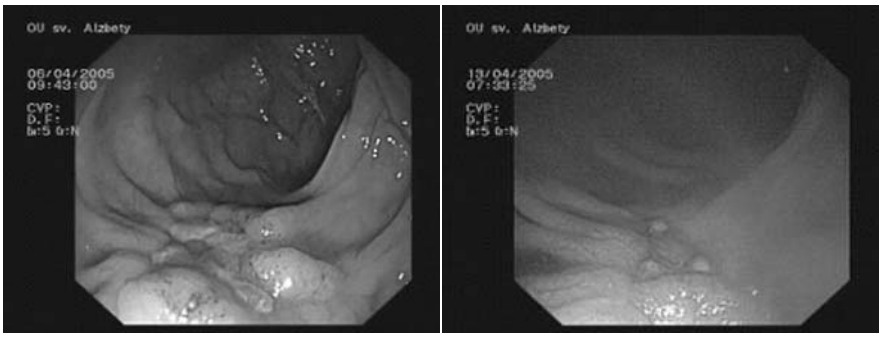

Figure 6. Endoscopic images of superficial intestinal dysplastic polyps before (left) and one week after (right) PDT.

In vivo fluorescence diagnostics and spectral analysis of biopsies, both relying on typical spectral properties of PpIX and other naturally occurring fluorophores in biological samples, were substantial part of the present study. Emission spectrum of PpIX is characterized by main peak at around 633 nm and the less intensive one around 700 nm [3]. As a result, excitation at 632.8 nm, which is laser line in our *in vivo* fluorescence measurements and is limited by the instrument does not allow us to detect the main emission peak of PpIX. Only emission and reflected light above 632.8 nm could be recorded. As a result, our spectra peaked at around 698 nm, consistently with an typical emission spectrum of PpIX.

PpIX is a natural component of the cell which is present also in the healthy tissue. However, after administration of ALA PpIX is produced to lower amounts in healthy tissue as compared to areas with higher synthesis typical for precancerous tissue [4]. It is therefore expected that emission spectra of these two types of tissues will differ only in intensity, consistent with our *in vivo* fluorescence measurements. Still, in fluorescence spectra of cryoslits from biopsies of suspicious area we detected additional emission band at around 675 nm. In healthy tissue no such band could be seen, there was only typical band of PpIX around 700 nm. The appearance of 675 nm band could be caused by photobleaching of PpIX and its conversion to photoprotoporphyrin PPp by acting light [3]. Phototransformation is indeed characterized by decreasing of fluorescence intensity at 633 nm and increasing intensity at 675 nm [10]. This transformation was caused most likely during the preparation of cryoslits from biopsies by acting of ambient sunlight, because during the endoscopy we did not observe creation of PPp in fluorescence spectra and also after PDT procedure. So this fact did not affect the PDT procedure. Our statetment confirmed fluorescence measurements of culture cells incubated with 1 mM ALA for four hours. These were exposed to radiation of endoscopic light source during the time interval equivalent for endoscopic treatment till biopsies removing i. e. from 15 sec to 30 sec. The other one was exposed to sunlight for 15 minutes what is time interval relevant for cryoslits preparation (Fig. 7). From these spectra is clear seen, that PPp is created only in the sample exposed to sunlight for 15 minutes.

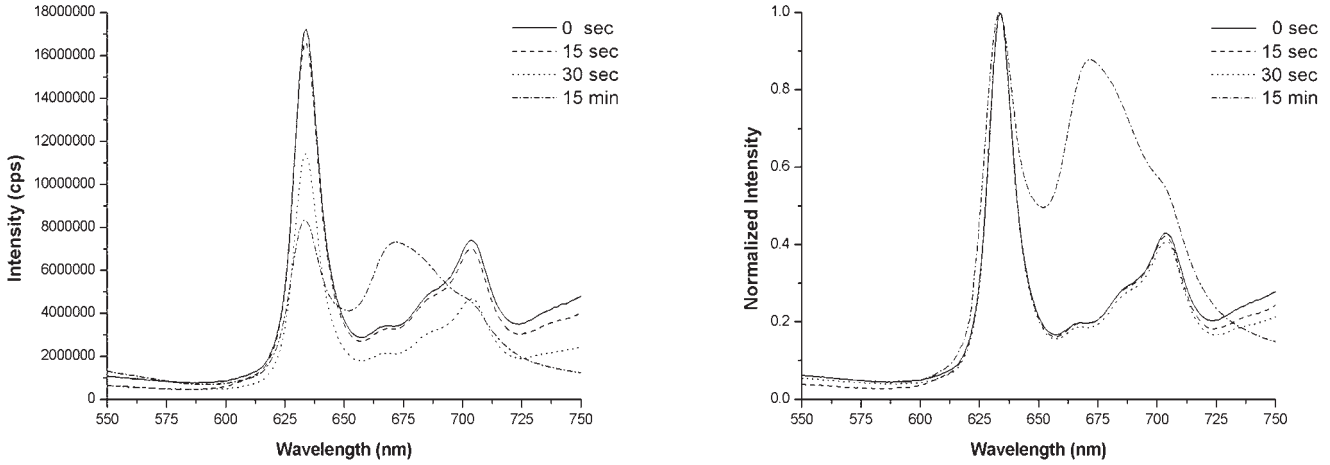

Figure 7. Fluorescence spectra (right – normalized at 633 nm) of culture esophageal cell lines incubated with 1 mM ALA for 4 hours. The samples were exposed to radiation of endoscopic light source for 0 sec., 15 sec., and 30 sec. and to sunlight for 15 min, respectively.

Contrary to *in vivo* fluorescence measurements, the choice of the excitation light for fluorescence microscopy was not limited to the red spectral region. Light of 458 nm was used in addition to 633 nm, giving us opportunity to detect a broader spectral range of fluorescence emission. Generally, spectral images of biopsies obtained after the excitation by 458 nm are superpositions of tissue autofluorescence and signal originating from accumulated PpIX. Autofluorescence in green region of spectrum is caused mainly by flavins and flavoproteins like e.g. FAD, NADH, LipDH, which play important role in mitochondrion respiratory chain [9]. Application of a method of linear unmixing to analyze spectral images of biopsies resulted in a separation of autofluorescence signal originating from flavins and flavoproteins from fluorescence signal of accumulated PpIX. Resulting unmixed images showed that essential part of red fluorescence occured due to accumulated PpIX. Moreover, our results from fluorescence microscopy showed differences in accumulation of PpIX in suspicious area of tissue as compared to healthy tissue. Spectral images demonstrated that PpIX is accumulated in the tissue only in discrete places, probably places with higher synthesis. No such areas with significant red fluorescence could be detected in cryoslits from healthy tissue. This supports an assumption of higher production of PpIX only in suspicious areas. In many cases the control of photosensitizer accumulation was performed and determined only from biopsies after PDT procedure and in number of cases the whole treatment had to be repeated because of low level of accumulated photosensitizer in treated area [1, 6, 12, 13]. Application of *in vivo* fluorescence diagnostics, such as used in the present study, clearly provides much better control of drug accumulation and therefore possibility to modify the treatment regime.

To summarize, differences in fluorescent spectra of PpIX in suspicious areas and in the healthy tissue suggest that this photosensitizer is suitable agent for fluorescence diagnostics of the precancerous tissue. Spectrally resolved fluorescence microscopy gives more detailed picture of accumulation and production of PpIX in tissue. Finally, ALA-based PDT is a suitable treatment modality for superficial precancerous lesions of esophagus and intestine.

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