VILNIUS UNIVERSITY

ROMUALDAS RUDYS

DETECTION OF ENDOGENOUS PORPHYRINS BY MEANS OF SPECTROSCOPY AND MICROSCOPY IN THE CASE OF RHEUMATOID ARTHRITIS

Summary of doctoral dissertation
Biomedical sciences, biophysics (02 B)

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VILNIAUS UNIVERSITETAS

ROMUALDAS RUDYS

ENDOGENINIŲ PORFIRINŲ DETEKCIJA SPEKTROSKOPIJOS IR MIKROSKOPIJOS METODAIS REUMATOIDINIO ARTRITO ATVEJU

Daktaro disertacijos santrauka

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ABBREVIATIONS

AIA – Antigen induced arthritis

ALA – 5-aminolevulinic acid hydrochloride

ALA-Me – Methyl 5-aminolevulinate hydrochloride

CE – Cartilage explants

DMEM – Dulbecco's Modified Eagle's medium

EP – Endogenous porphyrins

FBS – Foetal bovine serum

FLIM – Fluorescence-lifetime imaging microscopy

FWHM – Full width at half maximum

HA – Sodium hyaluronate

HE – Hematoxylin and eosin stain

i.a. – Intaarticular

i.v. – Intravenous

OA – Osteoarthritis

OVA – Albumin from chicken egg

PBS – Phosphate-buffered saline

PDT – Photodynamic therapy

PD – Photodiagnosis

PpIX – Protoporphyrin IX

RA – Rheumatoid arthritis

ROI – Region of interest

SD – Standard deviation

TCSPC – Time-correlated single photon counting

CONTENTS

1. INTRODUCTION

Optical properties of fluorescent compounds (fluorophores) serve as a powerful means to achieve optical molecular contrast using various instruments including spectrophotometers, microarrays, microscopes, and endoscopes. Fluorescence measurements can provide information not only about specific molecular makeup of a sample but also on the local environment surrounding the fluorophore. Distinct species of fluorophores may be characterized according to their excitation and emission spectra, their quantum efficiency, their polarization and their fluorescence lifetime[1-3]. The most common endogenous fluorophores used for the characterization of biological tissue include aromatic amino acids (tyrosine, tryptophane, and phenylalanine), structural proteins (elastin, collagens and collagen cross-links), enzyme metabolic co-factors (reduced nicotinamide adenine (phosphate) dinucleotide (NAD(P)H) and flavin adenine dinucleotide (FAD)), lipid components and porphyrins, absorption and emission characteristics of which have been extensively studied and rewieved[1-6]. Both steady-state and time-resolved fluorescence measurements can be employed for receiving quantitative and qualitative information with respect to composition and pathophysiology of biological tissues[1-8]. Autofluorescence-based contrast has been of particular interest in clinical research studies as the use of endogenous fluorescence for tissue characterization does not require the administration of a contrast agent. Also, techniques based on autofluorescence detection are minimally invasive and highly sensitive. The steady-state fluorescence technique, characterized typically by relative simple implementation and rather inexpensive instrumental setups, has been the most explored as a clinical tool for the diagnosis of diseased tissues *in vivo*. The sensitivity of many techniques for detecting particular stages of diseases is high, but their specificity remains limited due to a number of factors emerging from the difficulty to carry out absolute emission intensity measurements *in vivo*[5]. Additional information about the tissue can be provided by exogenous fluorophores with well-known fluorescence properties such as porphyrin sensitizers used in photodiagnosis (PD) and photodynamic therapy (PDT)[9-12]. Time-resolved spectroscopy technique employs an alternative method to measure tissue fluorescence that is not sensitive to the factors affecting signals of steady-state techniques. The fluorescence lifetimes reflect properties of the fluorophores

in biological tissues even if their fluorescence spectra are broad and overlapping. Also, the fluorescence lifetime of fluorophores varies with the molecular environment, but is usually independent of fluorophore concentration. Despite recognized inherent advantages, the full informative potential of fluorescence lifetime has not been extensively exploited in clinical settings due to several barriers, such as the complexity of the instrumental setup, the lengthy data acquisition and analysis, and the high instrumentation cost[5]. Numerous time-resolved fluorescence studies were conducted in excised tissue specimens *ex vivo*, however, due to changes in tissue biochemical composition related to functional properties (metabolism, hypoxia) and tissue morphology, the outcome of these studies cannot be fully extrapolated to *in vivo* measurements[3, 6, 8, 13]. Therefore, new techniques have been developed to investigate biological tissues in conditions, which closely resemble *in vivo* ones, and to perform time-resolved measurements *in vivo*[3, 6]. Since fluorescence spectroscopy data cannot be directly related to the morphological structure of biological tissue, one has to use a microscopic tissue analysis, which still remains the gold standard for identifying tissue types or a diagnosis. Recent introduction of confocal and multiphoton laser scanning microscopes initiated a breakthrough in biomedical fluorescence imaging[14, 15]. Tissue autofluorescence and fluorescence of exogenous fluorophores can be detected using fluorescence microscopy, but different endogenous fluorophores often cannot be distinguished due to overlapping fluorescence spectra. Fluorescence lifetime microscopy (FLIM), in which every single pixel in an image represents a fluorescence lifetime of endogenous fluorophores, gives an ability to resolve overlapping fluorescence spectra[3, 5, 6, 16]. FLIM has been also applied to investigate properties and transformations of sensitizers used in PDT[17-20]. These studies are very important in order to precisely assess PDT progress in real time and to prediction of adverse PDT effects on tissues. In searching for new treatment modalities for rheumatoid arthritis (RA), PDT has been considered as one of possible strategies[21-28]. Since the inflamed synovium in the case of rheumatoid arthritis, a chronic inflammatory disease of the joints, exhibits many features typical for neoplastic tissue such as hypermetabolic activity and extensive neovascularisation, there are numerous attempts to employ endogenous porphyrins for diagnostic and therapeutical purposes in rheumatology as well. The principle of PDT for the treatment of arthritis relies on the selective destruction of the inflamed synovium

caused by cytotoxic reactions involving reactive oxygen species that are generated in the inflamed cells after photoactivation of a sensitizer. Clinical PDT can be performed with exogenous photosensitizers or precursors, such as 5-aminolevulinic acid or its derivatives, which are converted into the endogenous photosensitizer protoporphyrin IX during haem synthesis. Since photo-induced damage during PDT occurs only in the vicinity of photosensitizer, it is essential, from a prognostic point of view, to determine the composition and distribution of photosensitizers in the tissues. When endogenous porphyrins can be detected by means of spectroscopy and microscopy methods, it is not enough to rely on measurements in only one spectral or lifetime dimension, since combination of several fluorescence detection methods makes it possible to improve the specificity of the fluorescence measurement and to achieve a higher contrast between different fluorophores for diagnostic or other purposes.

1.1. Actuality and scientific novelty

Rheumatoid arthritis (RA) is characterised by persistent synovitis, systemic inflammation, and production of autoantibodies. In industrialised countries, rheumatoid arthritis affects 0.5–1.0 % of adults, with 5–50 new cases per 100 000 annually[29]. The cause of RA is unknown, but it is considered to be a multifactorial disease, resulting from the interaction of both genetic and environmental factors[30]. The prevalence of RA is about 3 times higher in women than in men. Uncontrolled active rheumatoid arthritis causes joint damage, disability, decreased quality of life, and cardiovascular and other comorbidities. Disease-modifying antirheumatic drugs reduce synovitis and systemic inflammation and improve joint function[29]. However, numerous unresolved difficulties still exist for people with rheumatoid arthritis, and continuing introduction of innovative treatments might help to overcome many of them. Long-term remission induced by an intensive, short-term treatment selected in accordance with biomarker profiles is the ultimate goal[29]. Thus, PDT has been considered as one of the possible modalities for RA treatment. The main aim of PDT application for synovectomy is the selective approach to synovial destruction and the prevention of damage to the cartilage. Resent findings showed that, besides inflamed synovium, endogenous porphyrins were also detected in cartilage tissues of rabbit rheumatoid arthritis model *in vivo*[23, 24] and chondrocytes *in*

vitro[31] after the application of ALA or its derivatives. On the ather hand, the relative resistance of the chondrocytes to 5-ALA-PDT in comparison with osteoblasts and cells derived from synovial tissues had been shown[31, 32]. Cartilage has a complex structure, which consists of chondrocytes and extracellular matrix, so it is very difficult to obtain the representative model of the cartilage tissues to evaluate accumulation of endogenous porphyrins (EP). Until now, there was no data about the induction of EP and their accumulation pharmacokinetics in human cartilage tissue and chondrons of patients with RA or OA after the application of ALA or its derivatives.

Another important natural component of cartilage and synovial fluid is hyaluronic acid (HA). It was reported that HA of a high molecular weight (MW) exhibits anti-angiogenic and anti-inflammatory properties, whereas low MW fragments (<100 kDa) have the opposite biological activity; they are inflammatory, immuno-stimulatory and angiogenic[33]. It is also known that MW and concentration of HA are decreased in osteoarthritis and rheumatoid arthritis[34, 35]. Thus, the HA preparation of intermediate MW (GO-ON®), the clinical efficiency of which had been documented[36], was chosen to test its impact on the sensitization of cartilage. In order to examine and compare the capacity of cartilage explants and chondrons of OA patients to induce PpIX from ALA and its methyl ester (ALA-Me), the accumulation of endogenously produced PpIX has been also studied in synoviocytes of patients with RA and OA, which represent diseases of inflammatory and degenerative genesis, respectively.

This study also compared pro-sensitizing properties of exogenous ALA and ALA-Me after different administration routes by performing *in vivo* and *ex vivo* superficial fluorescence measurements of the endogenous porphyrins accumulated in the knee joint tissues of rabbits with AIA. For the first time ALA-Me was used *in vivo* to induce PpIX synthesis more selectively in the inflamed synovium. These results contribute to the development of a more selective synovectomy in RA based on the photodynamic therapy concept.

Several fluorescence detection methods have been combined to study the accumulation of endogenous porphyrins in the sensitized tissues and to distinguish structural features of the synovium and cartilage tissues. This is the first time the specimens of synovium

and cartilage tissues of healthy rabbits and rabbits with antigen induced monoarthritis were investigated by means of fluorescence spectroscopy, fluorescence intensity and lifetime microscopy, and the observed structural features in specimens were validated by applying the histopathological imaging based on conventional rapid haematoxylin–eosin (H&E) staining.

1.2. The aim of the study

To study accumulation and localization of endogenous porphyrins by means of spectroscopy and microscopy in experimental rheumatoid arthritis model and postoperative samples of patients after application of 5-aminolevulinic acid or its methyl ester.

1.3. Objectives

1. To compare the accumulation of endogenous porphyrins in synoviocytes, chondrons and cartilage explants of patients with rheumatoid arthritis or osteoarthritis after incubation with ALA or ALA-Me.
2. To investigate the impact of hyaluronic acid preparation on the induction of endogenous porphyrins in chondrons.
3. To compare the accumulation of endogenous porphyrins in rabbit experimental AIA model *in vivo* after intravenous and intraarticular injections of ALA or ALA-Me.
4. To determine *evx vivo* the accumulation of endogenous porphyrins in synovium and cartilage tissues of rabbits with AIA after *in vivo* application of ALA or ALA-Me.
5. To determine the localization of endogenous porphyrins in synovium and cartilage tissues.

1.4. Defended statements

1. RA and OA synoviocytes accumulate from 5 to 10 times more PpIX than cartilage explants and chondrons during 24 hours of incubation with ALA or

ALA-Me. RA and OA synoviocytes accumulate the same levels of PpIX after incubation with these precursors.

2. ALA and ALA-Me equally induce PpIX synthesis in specimens *in vitro* and *ex vivo*.
3. Preparations of hyaluronic acid boost PpIX accumulation in chondrons *in vitro*.
4. Fluorescence intensity of PpIX *in vivo* on the skin surface of inflamed knee joint of rabbit with AIA was up to 8 times higher after intraarticular 16 mM ALA injection than that after ALA-Me injection. The highest PpIX fluorescence intensity detected 2 hours after injection of precursors.
5. Fluorescence intensity of PpIX after intravenous 16 mM ALA injection *in vivo* on the skin surface of inflamed knee joint was about 5 times reduced in comparison with intraarticular injection, and almost undetectable after intravenous 16 mM ALA-Me injection.
6. Based on the fluorescence intensity of PpIX induced in the inflamed synovium, the injection of ALA-Me is about 5 times more effective than that of ALA. The cartilages of the inflamed and control knee joints of rabbits with antigen induced arthritis accumulate water-soluble porphyrins.
7. Three hours after intraarticular ALA-Me injection PpIX is localized in the cells of synovium and cartilage tissues.

2. MATERIALS AND METHODS

2.1. Preparation of cell specimens

Samples of synovium and cartilage were obtained as postoperative tissues during articular replacement surgery from patients with osteoarthritis (OA) and rheumatoid arthritis (RA) (Bioethical permission No 158200-12-270-63). OA patients were diagnosed according to radiological criteria; RA patients fulfilled the American College of Rheumatology revised criteria[37]. Synoviocytes were isolated from postoperative specimens of OA ($n_p = 6$) and RA ($n_p = 6$) synovial tissues. Experiments were performed on 200 000 cells per well, which corresponds to 95% of confluence. Cells were seeded into a 12 wells plate in a complete culturing DMEM for 12 h until incubation with 1 mM ALA (Sigma Aldrich Chemie, Miunich, Germany) or its methyl ester (ALA-Me) (Fluka Chemie GmbH, Hamburg, Germany). Non-adherent cells were removed and a medium was changed with a Tyroide's medium, which was prepared by dissolving 8 g NaCl, 0.2 g KCl, 0.2 g $CaCl_2$, 0.21 g $MgCl_2x6H_2O$, 0.05 g NaH_2PO_4, 1 g $NaHCO_3$ and 1 g glucose in 1 dm^3 of deionized water without serum and antibiotics. After incubation with prosensitizers the medium was removed, cells were washed three times with PBS (Phosphate-buffered saline) and scraped cells were resuspended into 20 µl volume. The counting of cells was performed in control wells by suspending the cells in CASY®ton, an electrolyte developed specifically for cell counting, and using a cell counter CASY® (Roche Innovatis AG). Fluorescence spectra of sensitized and control synoviocytes were measured in suspensions after 2, 4, 8 and 24 hours of incubation with ALA or ALA-Me.

2.2. Cartilage specimens

Pieces of cartilage were dissected from joint tissues of patients with OA ($n_p = 6$), washed with PBS and were cut into cartilage explants (CE) of 2-3 mm on each side, which were kept in an incubator at 37°C in a serum free medium (DMEM, 1 g / dm^3 glucose and antibiotics) before the experiments. Cartilage specimens were incubated with 1 mM ALA or ALA-Me for 2, 4, 8 and 24 hours. Later the incubation medium was removed and CE were washed with PBS before spectroscopic measurements.

2.3. Isolation of chondrons

Cartilage explants of patients with OA (n_p = 9) were used for the isolation of chondrons (a group of cartilage chondrocytes surrounded by a common membrane filled with extracellular content). Pieces of cartilage were dissected from patients' joint tissues, washed with PBS and cut into pieces of 1-3 mm on each side. About 7 g of CE were used for every experiment on chondrons. For isolation of chondrons CE were incubated (at 37°C and 5 % CO_2) for 12 h in a collagenase II solution (545U/ml) under conditions of constant shaking. 10 ml of collagenase II solution (545U/ml) (Biochrom AG: C2-22) were prepared for each 1 g of CE. After incubation chondrons were centrifuged for 5 min. at 400 *g* to remove a supernatant. Then chondrons were resuspended into 1 ml of a Tyroide's medium (without serum and antibiotics) and counted under a microscope using Fast-read 102 disposable slides for cell counting (Biosigma: BVS100). Later separated chondrons in a Tyroide's medium were seeded into 12-well plates ($2*10^5$ chondrons per well) and incubated for 12 h until treatment with ALA. To study EP accumulation chondrons of each patient (n_p = 3) were divided into a control group and an experimental group (in duplicates), which were incubated with Tyroide's or treated with 1 mM ALA for 2, 4, 8, and 24 h. For experiments with hyaluronic acid chondrons (n_p = 6) were divided into 2 groups (in duplicates): one incubated with 1 mM ALA served as control and the other was incubated with 1 mM ALA and 0.25% intermediate MW (800-1500 kDa) preparation of sodium hyaluronate (HA) (GO-ON®, Rottapharm, Ireland) for 12 h. After incubation the medium was removed, chondrons were washed three times with PBS and were resuspended into 20 µl volume for spectroscopic measurements. All incubation procedures were performed under dim light.

2.4. Animals

Twenty eight clinically healthy adult (about 3.5 kg body weight) male grey chinchilla rabbits involved in the study were kept under standard housing conditions. The animals were housed one per cage with rabbit chow and tap water *ad libitum* at the standard conditions and cared for in accordance with the European Convention and Guide for the Care and Use of Laboratory Animals and the Lithuanian laws. During the experiments rabbits were fed with oat grains to minimize the interference of fluorescence originated from chlorophyll degradation products. Antigen induced arthritis (AIA) was induced in

the right knee of animals following the procedures described in [23]. The intraarticular injection of OVA (day 0) (Munich, Germany) produced initial acute arthritis, which developed into chronic destructive inflammation in several days. The severity of progressing arthritis was determined by measuring the swelling of affected and control joints with a pair of callipers. The depilation of rabbit knees was performed 5 days before the spectroscopic measurements using depilatory cream for normal skin ("Veet", Dansom Lane Hull, United Kingdom). The study was approved by the Lithuanian Laboratory Animal Use Ethical Committee under the State Food and Veterinary Service (No 0218).

2.5. *In vivo* application of prosensitizers

After the induction of AIA rabbits were randomly divided into 3 groups. Based on the clinical signs of the joint swelling the application of ALA (Munich, Germany) or ALA-Me (Fluka Chemie GmbH, Germany) was performed not earlier than 3 days after the injection of OVA. 12 rabbits of the first group received 1 ml of 16 mM ALA (2.69 mg/ml, i.e. 0.9 mg/kg) solution either intraarticularly (i.a.; n = 7) or intravenously (i.v.; n = 5). 12 rabbits of the second group were injected with 1 ml of 16 mM ALA-Me (2.92 mg/ml, i.e. 0.97 mg/kg) solution (i.a.; n = 9 and i.v.; n = 3). The rabbits of the third group (n = 4) were not treated with ALA or ALA-Me and served as control.

2.6. Preparation of sensitized specimens for microscopy

The microscopy and spectroscopy of endogenously produced porphyrins were performed *ex vivo* by measuring fluorescence spectra of synovium and cartilage specimens taken from healthy and sensitized rabbit knee joints. Specimens of the inflamed joint were taken 3 hours after intraarticular injection of ALA-Me. Then healthy and sensitized specimens were immediately frozen with Solidofix®-Cryo spray (Carl Roth GmbH + Co. KG). Frozen tissues were microsectioned with a cryotome Leica CM1100 (Leica Biosystems, Germany). Frozen sections of 15 µm thick were collected on Histobond®+ (Carl Roth GmbH + Co. KG) glass-slides and air-dried at room temperature. The same 15 µm–thick cryostat sections were studied by means of fluorescence intensity and lifetime microscopy, as well as spectroscopy techniques. Later the morphological structures of those samples were distinguished by express staining with haematoxylin 2 and eosin (Fisher Scientific, UK).

2.7. Statistics

Samples of synoviocytes, CE and chondrons were prepared in duplicates for every measured incubation time point. Five spectral measurements were performed for each sample of suspension or CE. EP fluorescence intensity values obtained from fluorescence spectra were used to calculate the average fluorescence intensity value for each patient. These values were further averaged calculating the average value of an experimental group.

Generally, three zones on the surface of skin (overlying inner, outer and central parts of both control and inflamed knee joints) were used to register the fluorescence spectra at preset time intervals for every rabbit during *in vivo* experiments. The fluorescence intensity values of endogenous porphyrins were obtained by subtracting the autofluorescence background from the fluorescence spectrum measured after administration of prosensitizer. Analogously, four registered fluorescence spectra of each tissue specimen were used to calculate EP fluorescence intensity values during *ex vivo* experiments. Later, EP intensity values obtained from fluorescence spectra were used to calculate the average fluorescence intensity value for each rabbit *in vivo* and each specimen *ex vivo,* which were used to calculate the average value of an experimental group.

To evaluate the differences between EP fluorescence intensities after ALA and ALA-Me application in a single experimental group the Wilcoxon signed-ranks test was used. To evaluate the differences between EP fluorescence intensities after ALA and ALA-Me application in independent experimental groups the Wilcoxon rank-sum test (also known as the Mann-Whitney U test) was used. Data were expressed as mean ± SD, showing confidence intervals corresponding to a confidence level of 95%. P values less than 0.05 were considered to be significant. Software Origin v.8 was used for statistical calculations.

2.8. Fluorescence spectroscopy

The accumulation of EP in synoviocytes and chondrons was determined by measuring fluorescence spectra of $2 \cdot 10^5$ scraped cells resuspended into 20 µl of PBS in a glass tube. The spectra of endogenous porphyrins were obtained by subtracting the autofluorescence background of control specimens (normalizing intensity at 580 nm) from the

fluorescence spectra measured after administration of prosensitizers. Values of EP fluorescence intensity were measured at the main peak (e.g. at 635 nm for PpIX). The analogous calculations were applied to obtain EP fluorescence intensity values of CE specimens.

The kinetics of the accumulation of endogenously produced porphyrins *in vivo* were studied by measuring fluorescence spectra on the surface of the skin of rabbit knees hourly up to 4 hours after the injections of either ALA or ALA-Me. Then 25 rabbits (21 treated and 4 control) were sacrificed and both synovium and cartilage specimens were taken from left and right joints for the spectroscopic detection of porphyrins accumulated in articular tissues.

A custom-made spectroscopic device based on a fibre optics fluorescence spectrometer USB 2000-FL (Ocean Optics Inc., Dunedin, Florida, USA; 339-1026 nm) (FL) was used for fluorescence measurements both *in vitro* and *ex vivo* (Fig. 1.). The excitation light from a LED (an emission peak at 403 nm, FWHM 20 nm) adapted into a lamp holder (L) LS-450-L (Ocean Optics Inc., USA) was focused to one end of a bifurcated bundle (SOMTA, Latvia), which consisted of a central fibre and six circularly arranged fibres of 200 μm each (BF). The central fibre in the tip of the bundle was set for excitation of the samples. The light emitted from the samples was collected and transferred through the six lateral fibres to the other end of the bundle and then with a pair of converging lenses (L_1 and L_2) was directed through a 500 nm longpass filter (F) ('ЖС 18', LOMO, Russia) to the tip of a fibre connected directly to the spectrometer.

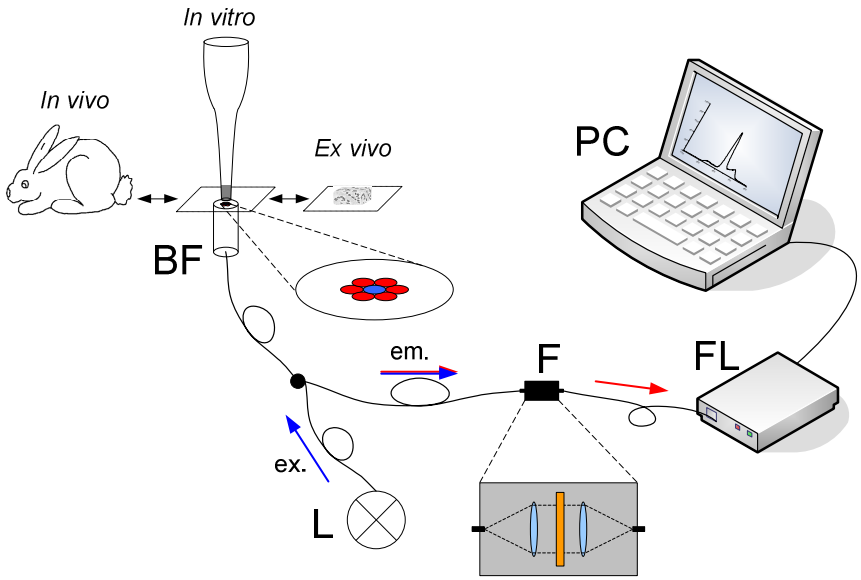


Fig. 1. Scheme of the experimental set-up for fluorescence measurements

2.9. Fluorescence microscopy

Tissue specimens were imaged by means of a confocal fluorescence microscope “Eclipse TE2000” (Nikon, Japan) with a confocal scanning C1si system performing sequential scanning with a beam of a diode laser (405 nm), at 40x total magnification using a 40x NA 0.95 Plan Apo objective. The three-channel RGB detector (band-pass filters: 500-590 nm and 621-755 nm for green (G) and red (R) channels, respectively) was used for standard images. Image processing was done using the “Nikon EZ-C1 Bronze v.3.80” and the “ImageJ 1.43” software.

A pulsed diode laser emitting at 405 nm (PDL 800-B, PicoQuant GmbH, Berlin, Germany) was coupled to a laser scanning microscope Nikon “Eclipse TE2000” to measure the lifetimes of tissue autofluorescence and sensitized tissue fluorescence. The pulse repetition rate was set to 20 MHz, which allowed using a measurement time interval of 50 ns. FLIM was performed using a time-correlated single photon counting (TCSPC) module PicoHarp 300, and 3200 channels of a detector were used to register a fluorescence decay curve. A single channel SPAD detection unit was used for photon counting at 650/160 nm or 637/13 nm spectral ranges (FWHM) by changing filters. Each fast FLIM image representing the average fluorescence lifetimes was obtained by collecting 1000 counts at the peak value and the image resolution was set at 512x512 pixels. Registered fluorescence decay curves were fitted with exponential decay functions to obtain characteristic fluorescence lifetime values, which were used to calculate the FLIM images by means of the software SymPhoTime v.5.2 (PicoQuant GmbH, Berlin, Germany).

A spectrometer QE65000 (Ocean Optics Inc., USA) was connected to a Nikon “Eclipse TE2000” confocal scanning C1si system to measure integral fluorescence spectra during sequential scanning of samples with a beam of a diode laser (405 nm). The light emitted from the tissue was collected through a long pass filter (‘ЖС 18’, LOMO, Russia) and directed to the tip of a fibre connected directly to the spectrometer.

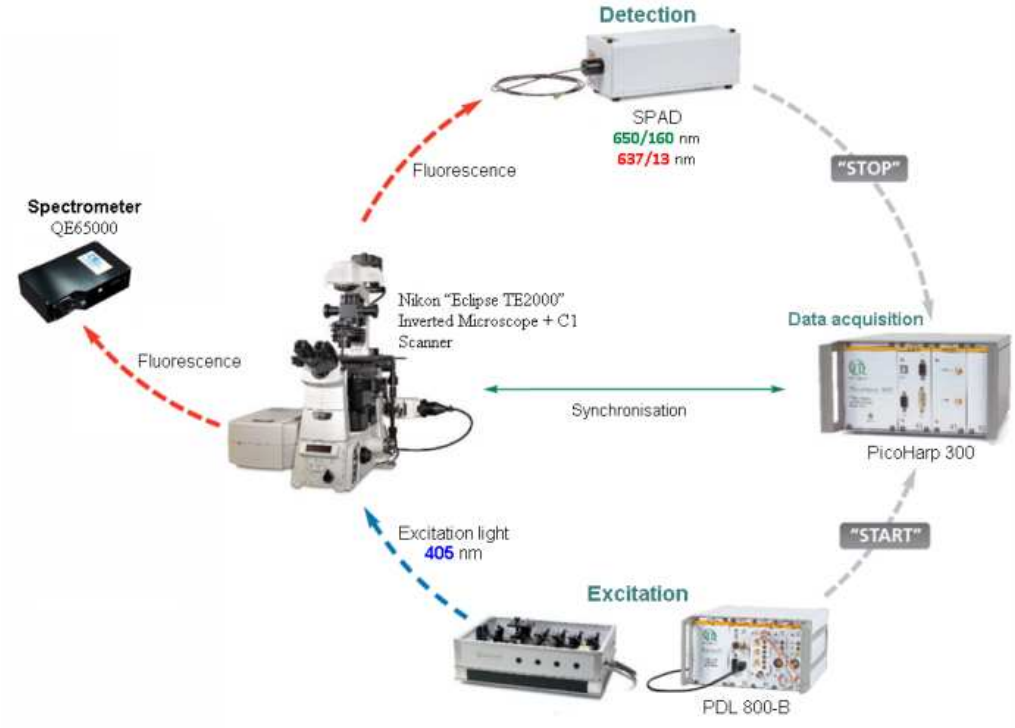


Fig. 2. Measurement system [adapted from www.picoquant.com]

3. RESULTS

3.1. Spectroscopic study of patients with RA or OA synoviocytes, chondrons and cartilage explants

To determine whether the dynamics of PpIX accumulation following administration of ALA or ALA-Me can be associated with the enhanced inflammation state in RA versus OA, the simultaneous sensitization experiments were performed on RA and OA groups of synovial cells. In addition, the accumulation dynamics of EP was followed in CE specimens and chondrons taken from patients with OA.

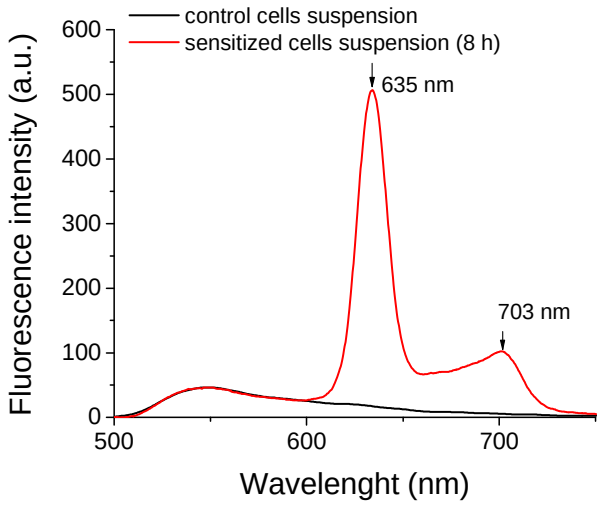


Fig. 3. Typical fluorescence spectra of control and sensitized (incubated 8 h with ALA or ALA-Me) synoviocytes suspended in PBS

Spectroscopic measurements confirmed that synoviocytes of patients with RA and OA have ability to accumulate the excess amounts of endogenous porphyrins after treatment with ALA and ALA-Me. Typical fluorescence spectra of control synoviocytes and synoviocytes incubated with ALA or ALA-Me in suspensions are presented in Fig. 3. Two distinct bands in the fluorescence spectra above the autofluorescence background with peaks at 635 nm and around 700 nm clearly indicated the presence of endogenous protoporphyrin[38]. Fluorescence of endogenous porphyrins was not registered in suspensions of control synoviocytes during a whole observation period.

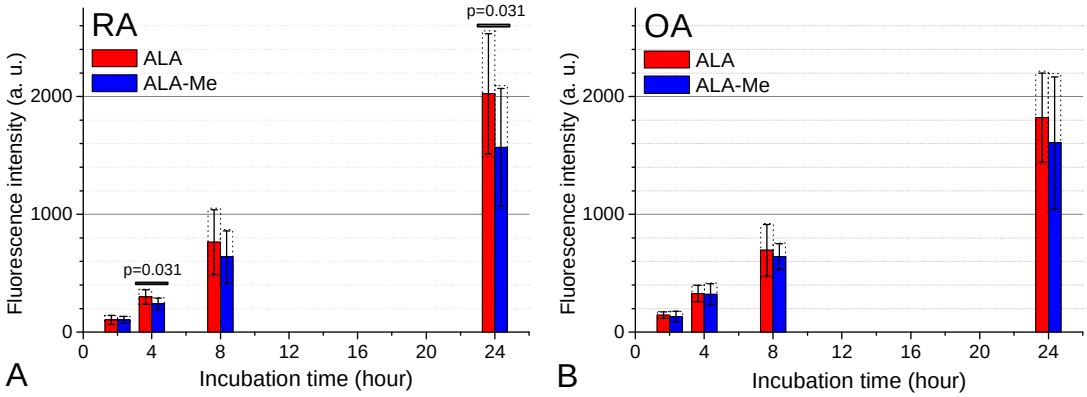


Fig. 4. Average peak fluorescence intensity (at 635 nm) values of PpIX at different times after incubation with 1 mM ALA or 1 mM ALA-Me measured in suspensions of synoviocytes isolated from synovium tissues of patients with RA (A, for each time point n_p = 6) and OA (B, n_p = 6), error bars – SD. Dotted bars represent confidence intervals (at 95%).

Fig. 4 shows time related changes in the averaged peak fluorescence values for RA (n_p = 6) and OA (n_p = 6) synoviocytes in suspensions. The average intensities of PpIX after ALA application were higher in comparison with ALA-Me at all time points, especially at 24 h. Furthermore, there was no significant difference ($p > 0.128$) between the averaged fluorescence intensity values of PpIX measured in RA and OA synoviocytes at the same time points. One has to mention that the notable variations both in the measured intensities and the accumulation dynamics have been observed between cell samples isolated from individual patients within the RA and OA groups (data not shown).

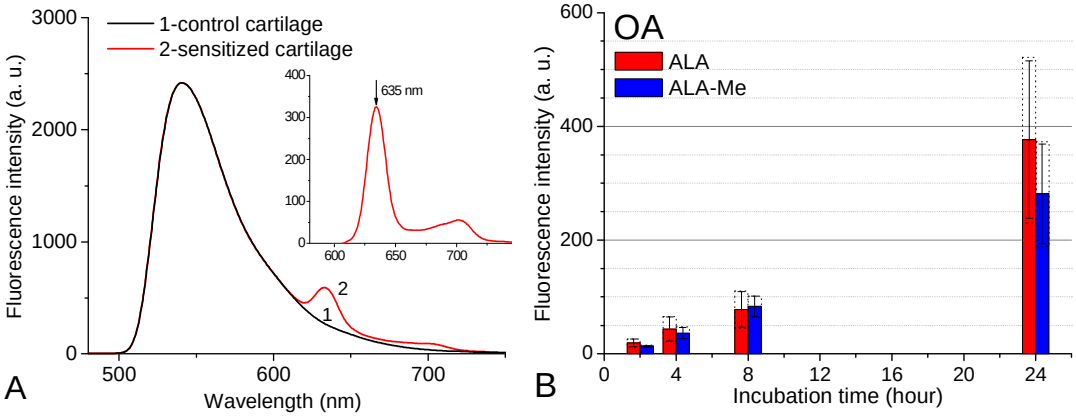


Fig. 5. A – typical fluorescence spectra of control (curve 1) and sensitized (curve 2, after 24 h incubation with 1 mM ALA) OA cartilage explants. An inset shows a subtracted spectrum of PpIX with a main peak at 635 nm; B – average values of PpIX fluorescence intensity (at 635 nm) measured *ex vivo* in CE (n_p = 6) at different time points after incubation with 1 mM ALA or 1 mM ALA-Me, error bars – SD. Dotted bars represent confidence intervals (at 95%).

Fig. 5, A shows a typical autofluorescence spectrum of control CE and a fluorescence spectrum of sensitized CE, in which the fluorescence bands of PpIX at 635 nm and at about 705 nm are clearly distinguishable. Average fluorescence intensity values of PpIX measured in cartilage explants of patients with OA (n_p = 6) at different time points after incubation with 1 mM ALA or 1 mM ALA-Me are presented in Fig. 5, B. There was no significant ($p > 0.184$) difference in fluorescence intensities after application of ALA or ALA-Me at all time points of observation period. However, it is seen that the PpIX intensity values in CE were five or more times lower in comparison with those obtained from synoviocytes ($p = 0.005$, at each time point). The slightly decreased values of this ratio were detected after 24 hours of incubation.

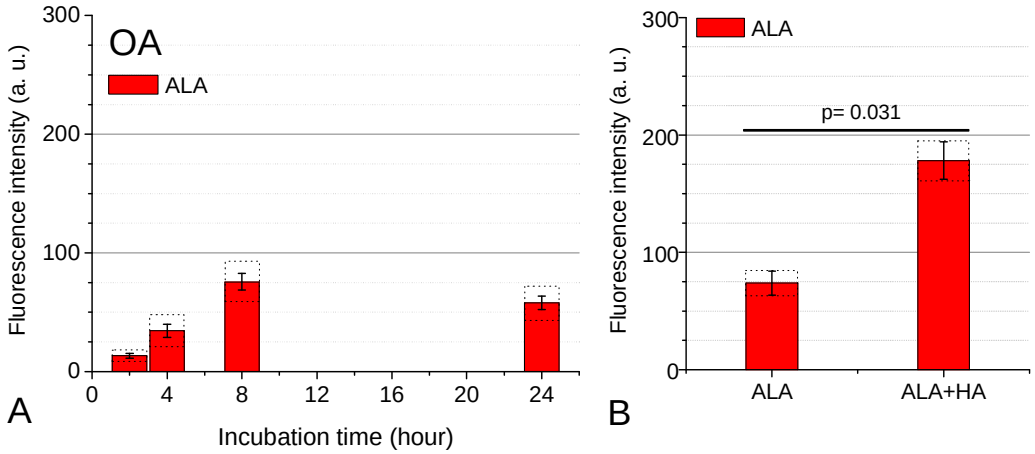


Fig. 6. Average fluorescence intensity values of PpIX (at 635 nm) measured at different times in suspensions of chondrons obtained from patients with OA: A – after incubation with 1 mM ALA (n_p = 3); B – after 12 h incubation with 1 mM ALA alone or adding 0.25 % sodium hyaluronate (n_p = 6), error bars – SD. Dotted bars represent confidence intervals (at 95%).

Taking into account the differences in preparation of CE and synoviocytes' specimens, the more detailed study of PpIX accumulation was performed on chondrons. Average fluorescence intensity values of PpIX measured at different times in suspension of chondrons (Fig. 6, A) were not significantly different ($p > 0.366$) from those values measured in CE (Fig. 5, B) up to eight hours of incubation. However, after 24 hours of incubation with ALA, the fluorescence intensities of PpIX diminished in suspended chondrons and became significantly ($p = 0.028$) lower than corresponding intensities in CE. According to the obtained ratios of fluorescence intensities, the specimens of chondrons from patients with OA accumulated about ten times less PpIX than the

synoviocytes (Fig. 4.). As expected, chondrons of the control group did not accumulate any endogenous porphyrins.

To find out whether the application of HA can affect the accumulation of endogenous porphyrins in chondrons, the experiments were performed adding the 0.25 % sodium hyaluronate to the incubation medium together with ALA, and chondrons were incubated for 12 h. The control group was incubated only with 1 mM ALA. The average PpIX fluorescence intensity values from the suspensions of chondrons (n_p = 6) are compared in Fig. 6, B. The Wilcoxon signed-ranks test, which was performed on the average PpIX fluorescence intensity values from specimens with HA and control ones after 12 h of incubation, showed significant (p = 0.031) difference between them.

3.2. Spectroscopic study of endogenous porphyrins in rabbit AIA model

3.2.1. *In vivo* spectroscopic evaluation of endogenously synthesized porphyrins

The averaged spectral data of the *in vivo* measurements are represented in Fig. 7. The fluorescence spectra that were measured after i.a. injection of ALA revealed significant difference between the inflamed and control joints. The autofluorescence intensity of rabbit knees decreases with increasing wavelength, while the fluorescence spectra of rabbit knee exhibit fluorescence bands with the peaks around 635 and 700 nm after ALA injection. The mean fluorescence intensity at 635 nm obtained in the spectra registered on the skin surface of the arthritic joints 2 hours after the injection of ALA was about five times higher than that in the spectra of the control joints. The typical fluorescence spectra of PpIX possess a dominant fluorescence peak at 635 nm with a second peak around 700 nm. Therefore, the spectral changes that were detected on the skin surface of rabbits after the injections of ALA or ALA-Me into the right (arthritic) knee could be assigned to the accumulation of the PpIX in the joints of rabbits. The comparison between the averaged fluorescence intensity of the inflamed knee joint and the fluorescence intensity of the control knee after the subtraction of the corresponding initial autofluorescence indicated the significant production of endogenous porphyrins, especially of PpIX, in the inflamed knee 2 h after i.a. injection of ALA. Whereas, the fluorescence intensity registered on the skin surface of the arthritic joint after the i.a. injection of ALA-Me was very weak (Fig. 7, B) and it was almost undetectable on the

control joint.

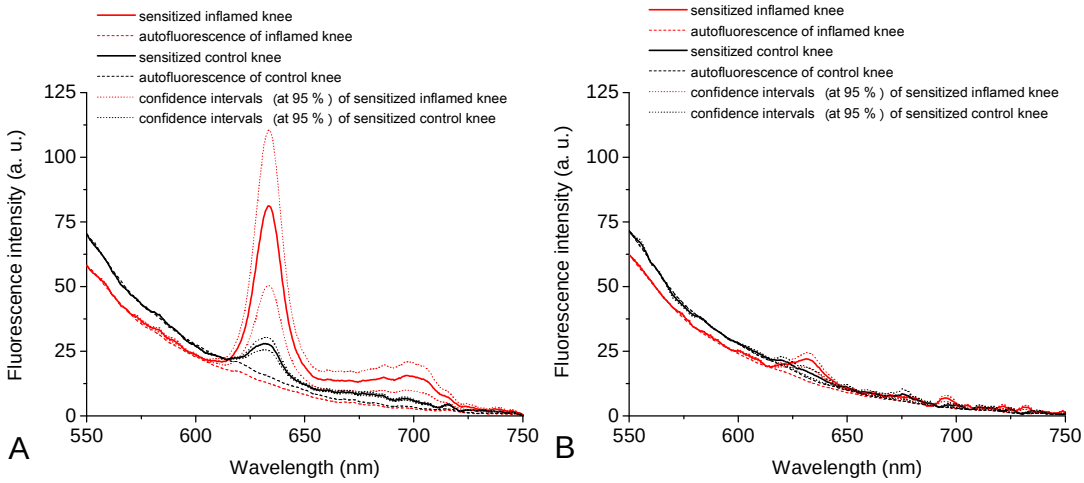


Fig. 7. The averaged fluorescence spectra measured on the inflamed (right) knee joint and the control (left) knee joint 2 h after i.a. injection of ALA (n=7) (**A**) and ALA-Me (n=9) (**B**). Dashed lines depict averaged autofluorescence of corresponding knees before the injection of prosensitizers. Dotted lines represent confidence intervals (at 95 %)

Fig. 8 shows the averaged values of the fluorescence intensity at 635 nm (after the autofluorescence background substraction) that were measured at different times after i.a. injection of prosensitizers ALA (A) and ALA-Me (B). The highest mean fluorescence intensity and the contrast between the inflamed and the control knee joints were observed two hours after the injection.

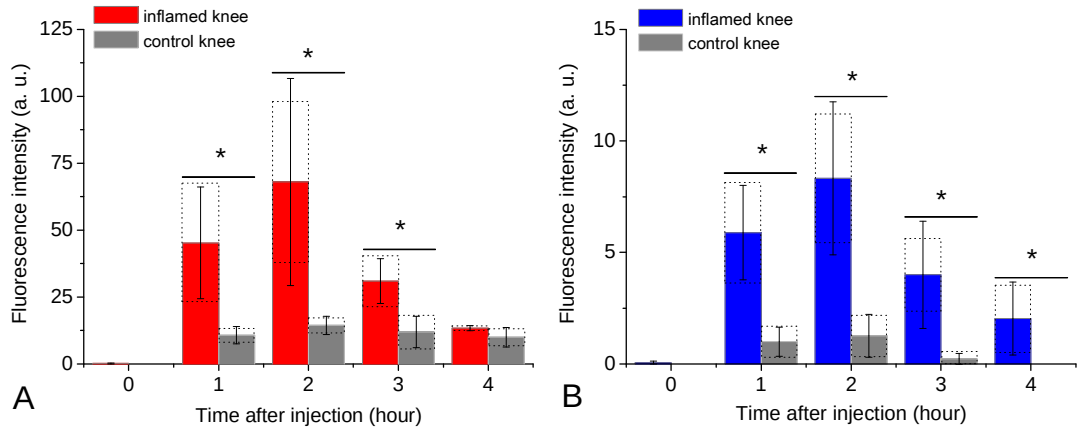


Fig. 8. Values of fluorescence intensity (mean ± SD) of PpIX (*in vivo*) averaged at different times after i.a. injection of ALA (a number of rabbits, n = 7) (A) and ALA-Me (n = 9) (B). * Fluorescence intensities of PpIX on the right knee differ significantly from those on the left knee, $P < 0.05$. Dotted bars represent confidence intervals (at 95 %)

The measurements of the fluorescence spectra performed *in vivo* on the skin of arthritic and control joints at 1 hour after the injection of ALA into an ear vein (i.v.) revealed a significant difference in fluorescence intensity at 635 nm, however, these intensities

were not significantly different at 2 h after the injection (Fig. 9, A). After the intravenous injection of ALA-Me endogenous porphyrin fluorescence was almost undetectable (Fig. 9, B).

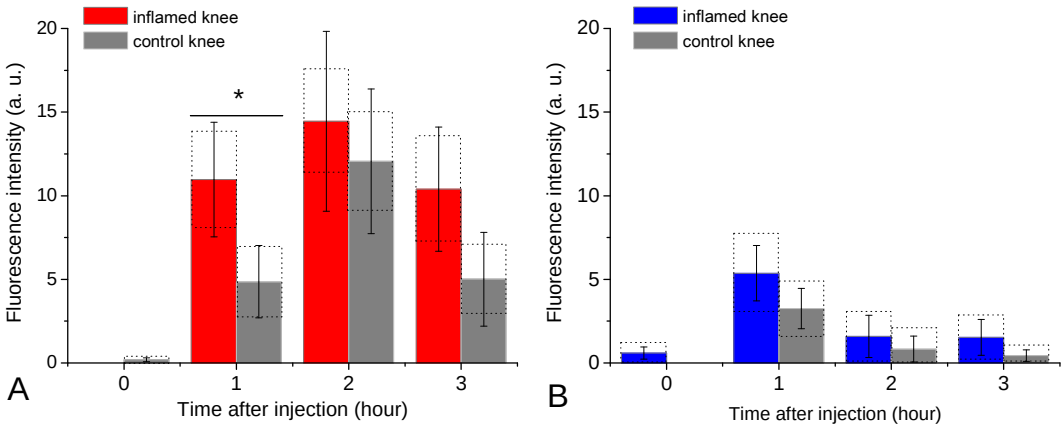


Fig. 9. Values of fluorescence intensity (at 635 nm, mean ± SD) of PpIX (*in vivo*) being averaged at different times after i.v. injection of ALA (a number of rabbits, n = 5) (**A**) and ALA-Me (n = 3) (**B**). * Fluorescence intensities of PpIX on the right knee differ significantly from those on the left knee, $P < 0.05$. Dotted bars represent confidence intervals (at 95 %)

3.2.2. *Ex vivo* spectroscopic assessment of endogenous porphyrins in tissues after ALA or ALA-Me injections

Four hours after the injection of prosensitizers the animals were sacrificed to take tissue specimens of synovium and cartilage for further spectroscopic studies *ex vivo*. The measurements were performed not later than 5 min. after the specimens were collected. Fluorescence spectra that were registered in tissues after i.a. injections of ALA or ALA-Me differed both in intensities and relative contribution of endogenous porphyrins (Fig. 10). The cartilage specimens had more than two fluorescence bands in the red spectral region reflecting several types of endogenous porphyrins induced after the injection of either ALA or ALA-Me. The fluorescence spectra of the cartilage in addition to the band of PpIX had another band with a peak around 620 nm and a region of increased fluorescence intensity at 650-680 nm corresponding to water-soluble uro- and coproporphyrins[23, 38, 39]. Meanwhile, the spectra of the synovium specimens contained only the fluorescence bands of PpIX.

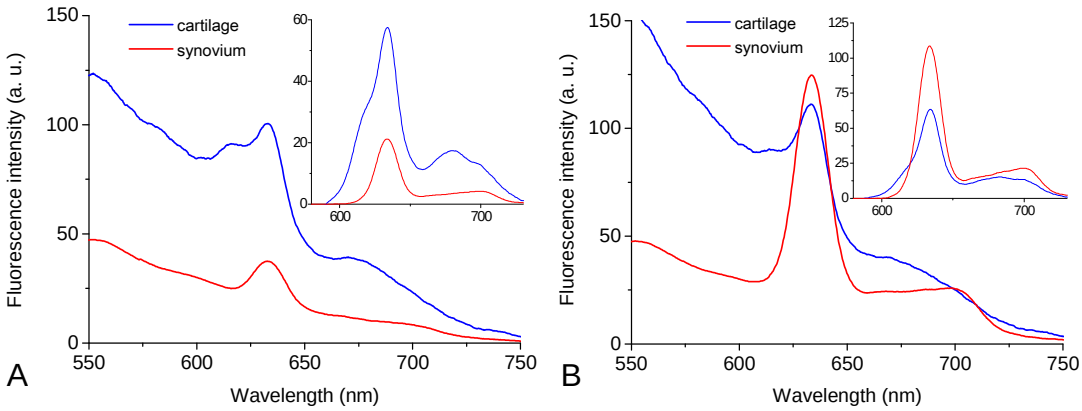


Fig. 10. Averaged fluorescence spectra of endogenously produced porphyrins in synovial and cartilage tissues of arthritic joint after i.a. injections of ALA (n = 7) (**A**) and ALA-Me (n = 6) (**B**). Insets: the fluorescence difference spectra that were obtained by subtracting the averaged autofluorescence spectrum (being measured on either synovium or cartilage of the control group animals) from the corresponding averaged spectrum registered on inflamed tissues

Comparison between the peak intensity values of the fluorescence spectra obtained after subtracting autofluorescence showed that after the i.a. injection of ALA the mean fluorescence intensity of PpIX in cartilage tissues of an arthritic joint exceeded that in synovium tissues more than two times (Fig. 10, A, inset). However, in the group that received ALA-Me the recorded average fluorescence intensity of PpIX was higher in the inflamed joint synovium in comparison with cartilage (Fig. 10, B, inset). According to experimental data, PpIX fluorescence intensity in arthritic synovium had increased about five times on average when ALA-Me was injected i.a. instead of ALA (Fig. 11). The i.v. injection of ALA or ALA-Me, however, induced a negligible PpIX fluorescence signal in all synovium tissue specimens, and no signal was detected in cartilage specimens taken from an arthritic joint (data not shown). In general, no PpIX fluorescence signal was registered in tissue specimens of control joints.

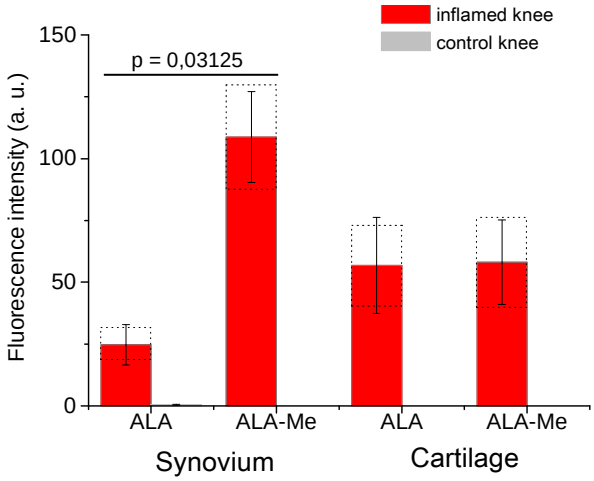


Fig. 11. PpIX fluorescence intensity mean ± SD (at 635 nm) in specimens *ex vivo* after i.a. injection of prosensitizers, n = 7 for ALA and n = 6 for ALA-Me. * Fluorescence intensities of PpIX in the inflamed synovium after ALA-Me application differ significantly as compared to ALA application, $P < 0.05$. Dotted bars represent confidence intervals (at 95 %)

As mentioned before, the fluorescence peak of water-soluble porphyrins (at ~ 620 nm) was registered in the spectra of cartilage specimens of both knee joints besides the fluorescence band of PpIX. Fluorescence intensity of these porphyrins in the cartilage specimens of both arthritic and control joints had similar values in all groups, only after i.a. injection of ALA (Fig. 12) the mean fluorescence intensity at 620 nm was about two times higher in the arthritic joint cartilage.

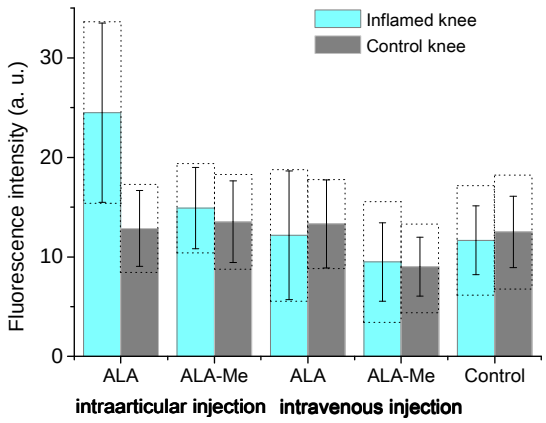


Fig. 12. Fluorescence intensity at 620 nm (mean ± SD) of water-soluble porphyrins in cartilage after i.a. injection of ALA (n = 7) or ALA-Me (n = 6) and i.v. injection of ALA (n = 5) or ALA-Me (n = 3), the control group n = 4. Dotted bars represent confidence intervals (at 95 %)

3.3. Study of endogenous porphyrins localization in sensitized tissues

Healthy cartilage

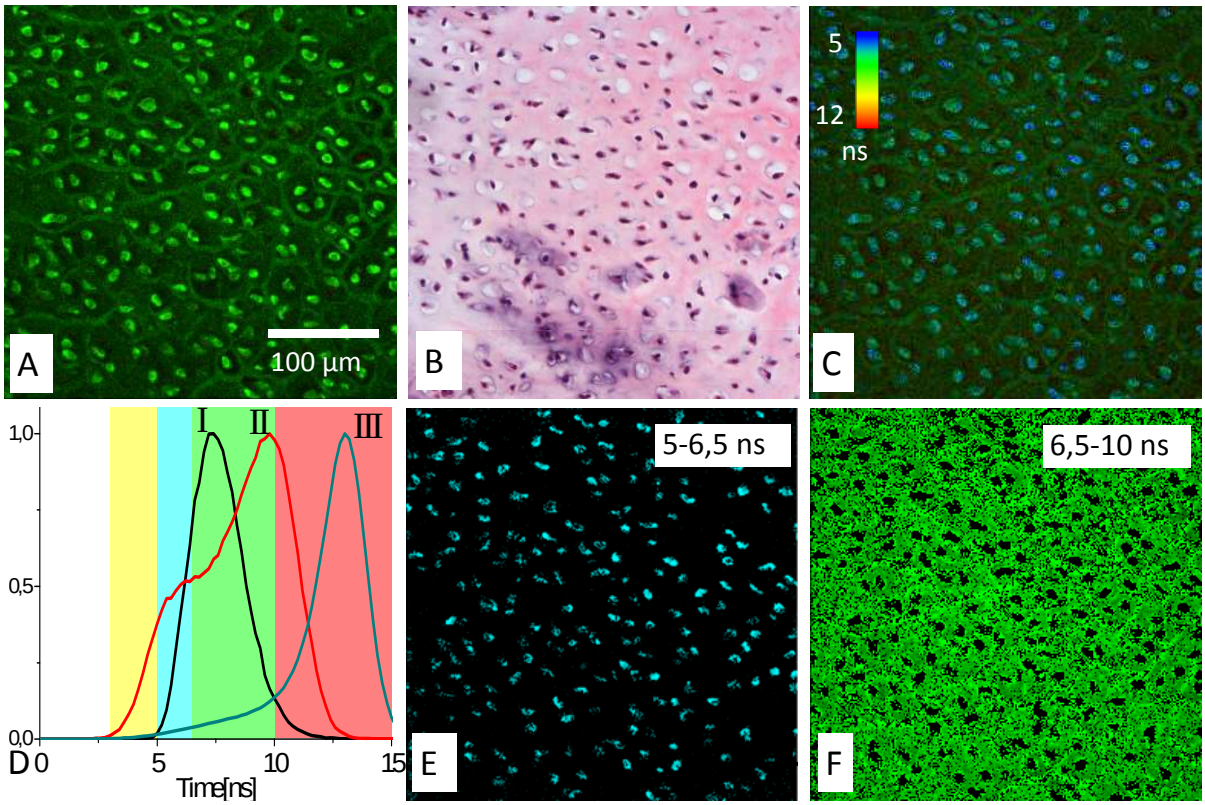


Fig. 13. Images of the healthy rabbit cartilage specimen: A – a fluorescence image taken combining G and R channels; B – a H&E stained histological image; C – a fast FLIM image in **650/160** nm spectral range; D – normalized distribution of average fluorescence lifetimes of healthy cartilage in **650/160** nm spectral range (curve I) as well as sensitized cartilage in **650/160** nm (curve II) and **637/13** nm (curve III) spectral ranges, time gates :yellow – 3-5 ns, blue – 5-6.5 ns, green – 6.5-10 ns, red – 10-15 ns ; E, F – time-gated fluorescence lifetime images (gates are shown on pictures). Total magnification 40x

Fluorescence intensity and lifetime microscopy images of the cartilage specimen of a healthy rabbit are presented in Fig. 13 together with corresponding histological images. The highest autofluorescence intensity of healthy cartilage was detected in the green/orange spectral region (520-590 nm), which falls into a green channel of the microscope, and no fluorescence was registered in the red spectral region (621-755 nm) (Fig. 13, A). From the histological image (Fig. 13, B) the tissue was defined as a healthy transitional layer of articular cartilage. Dark spots seen throughout the histological image are cells` (namely chondrocytes) nuclei and a light pink area is an extracellular matrix. The brighter green spots seen in the fluorescence image clearly correspond to the dark spots seen throughout the histological image (Fig. 13, B). Fast FLIM imaging (Fig. 13,

C) revealed that the lifetimes of intrinsic fluorophores of the healthy cartilage were limited between 5 ns and 10 ns, as indicated in the distribution of average lifetimes (Fig. 13, D, curve I). The cells of a healthy cartilage had shorter average lifetimes than extracellular matrix. Since fast FLIM technique allows to gate different average lifetimes, which then can be assigned either to particular fluorophores or their specific environment-sensitive states, a combined image has been split into several pictures depicting average fluorescence lifetime values in shorter intervals. As it is seen in gated images (Fig. 13, E, F), the specific structures distinguishable in tissue specimens at lifetimes between 5 ns and 6.5 ns (Fig. 13, E) correspond to cells, while the structures seen at lifetimes between 6.5 ns and 10 ns (Fig. 13, F) can be attributed to extracellular matrix when compared with a histological image.

Sensitized cartilage

Typical fluorescence spectra being registered *ex vivo* on the healthy and inflamed cartilage specimens taken *post mortem* from healthy and sensitized rabbits three hours after ALA-Me injection are presented in Fig. 14, A. Contrary to the spectra of the healthy rabbit specimens, the highest fluorescence intensity of sensitized cartilage was detected in the red spectral region, which falls into the range of the red channel of the microscope (620-700 nm). Two distinct bands in the fluorescence spectra clearly indicated the presence of endogenous porphyrins. The microscopic fluorescence imaging of the sensitized cartilage (Fig. 14, B) revealed only weak fluorescence in the red channel though. This fluorescence pattern very well corresponds with intratissual location of the cells identified from the histological image (Fig. 14, C). Besides the dark spots seen throughout the histological image that are cell nuclei and the light pink area resembling to extracellular matrix as in the control cartilage, a tangential zone of the cartilage with flattened chondrocytes is seen in the top of the image. Below it lays a transitional cartilage zone, while a calcified cartilage and a subchondral bone (dark area) are present in the bottom of the sample. No damage of cartilage is seen in the histological image.

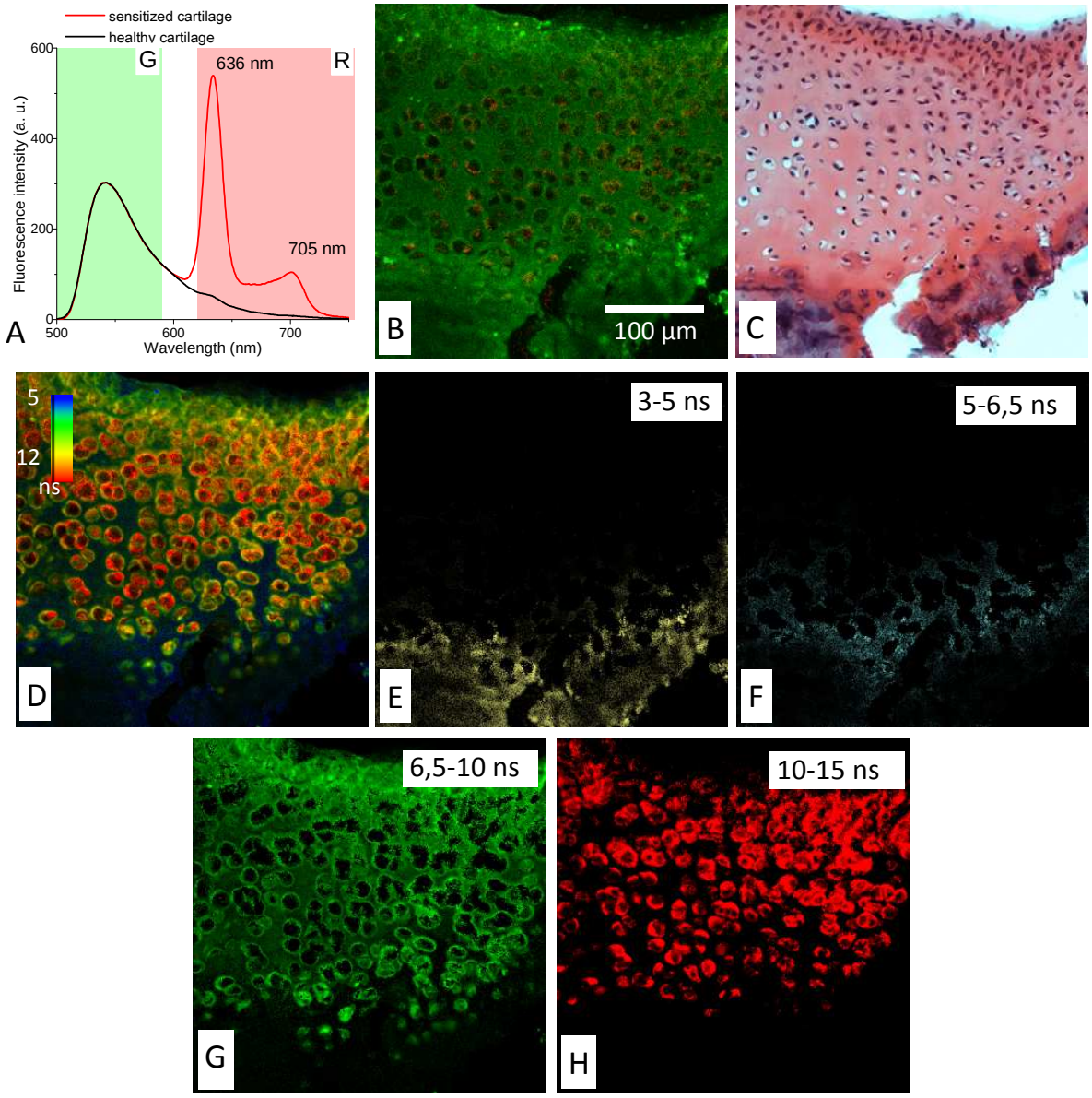
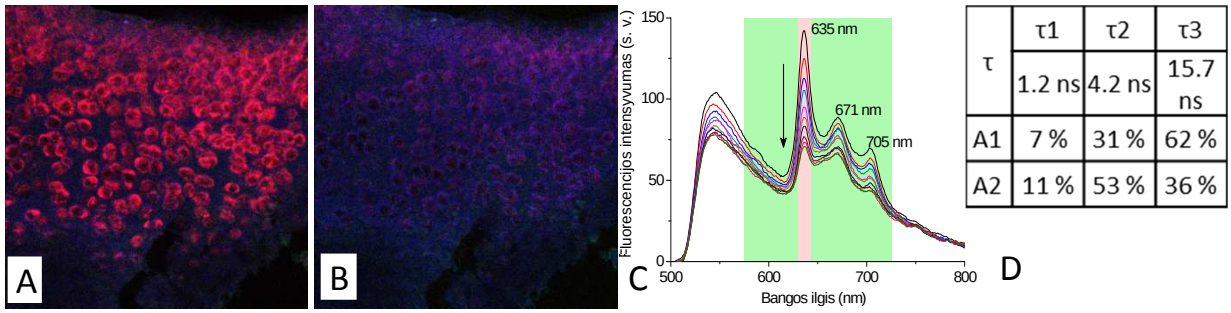


Fig. 14. Images of the sensitized cartilage specimen: A – normalized fluorescence spectra of healthy and sensitized cartilage, colour areas represent the spectral ranges of fluorescence microscope 'green' (G) and 'red' (R) channels; B – a fluorescence image taken combining G and R channels; C – H&E stained histological image; D – a fast FLIM image in **650/160** nm spectral range; E-H – time-gated fluorescence lifetime images (lifetimes gates shown on pictures). Total magnification 40x

Fig. 14, D represents a fast FLIM image of a sensitized cartilage, which is completely different from a healthy cartilage image with regard to emerged shorter and longer average lifetimes. The distribution of average lifetimes of fluorophores in the sensitized cartilage in 650/160 nm spectral range is limited between 3 ns and 13 ns (Fig. 13, D, curve II). The gating of fluorescence lifetime images (Fig. 14, E-H) revealed that the shortest lifetimes in the range of 3.5-5 ns belong to the fluorescing subchondral bone

structure (Fig. 14, E). The pattern of extracellular matrix (Fig. 14, G) can be distinguished at the same average lifetimes like in the healthy cartilage – between 6.5-10 ns (Fig. 13, F). However, contrary to the gated image of healthy cartilage, lifetimes between 5-6.5 ns cannot be attributed to the pattern of cells but to a very weak pattern of calcified cartilage. Instead, the cells of the sensitized cartilage can be distinguished in the image gated at longest fluorescence lifetimes – 10-15 ns (Fig. 14, H), and this pattern corresponds with location of cells in a histological image (Fig. 14, C) as well as with a red fluorescence pattern in a fluorescence image (Fig. 14, B). The fluorescence spectra of sensitized cartilage (Fig. 14, A) imply that the longest fluorescence lifetimes in the 650/160 nm spectral range are determined by endogenously synthetized protoporphyrin IX, which accumulates in cells.



τ	τ1	τ2	τ3
	1.2 ns	4.2 ns	15.7 ns
A1	7 %	31 %	62 %
A2	11 %	53 %	36 %

Fig. 15. FLIM images before (A) and after (B) photobleaching in **650/160** nm spectral range: green colour – τ1 = 1.2 ns, blue colour – τ2 = 4.2 ns, red colour – τ3 = 15.7 ns. Total magnification 40x. C – changing of sensitized cartilage fluorescence spectra during exposure to 405 nm light; D – calculated fluorescence lifetimes and its amplitudes before (A1) and after (A2) photobleaching

Fig. 15, A, B shows recalculated FLIM images of the sensitized cartilage before and after its extended exposure to the laser radiation. Photobleaching of a specimen was induced by scanning it repeatedly during ten minutes with a beam of a 405 nm laser connected to the scanning fluorescence microscope, the same system used for fluorescence imaging. The recalculated FLIM image of sensitized cartilage specimen revealed that the highest intensity of the fluorescence signal corresponding to the longest decay constant τ_3 was detected in the cells (Fig. 15, A). Comparison with the fast FLIM images also indicated the resemblance between the distribution patterns of fluorophores with longest average decay times (Fig. 14, H) and those of fluorophores with the highest magnitudes of τ_3 (Fig. 15), In addition, the calculated values for the fluorescence intensity of τ_3 were lower in the areas, where the average fluorescence decay times were

shorter. Decreased fluorescence bands of PpIX and an increased fluorescence band of a photoproduct were seen in the fluorescence spectra (Fig. 15, C) registered after the exposure of specimens to 405 nm radiation. The FLIM images calculated after exposure also strongly differed from those calculated for unexposed areas. This difference is caused only by the different inputs of the magnitudes of three calculated decay times. The highest magnitude before exposure was of τ_3, while after exposure that of τ_2 took the lead (Fig. 15, D). Assuming that the longest fluorescence lifetimes are determined by PpIX, its photodegradation leads to decreased input of the longest lifetime magnitude, since PpIX is very unstable, especially, when exposure is at the absorption region of the Soret band.

Healthy synovium

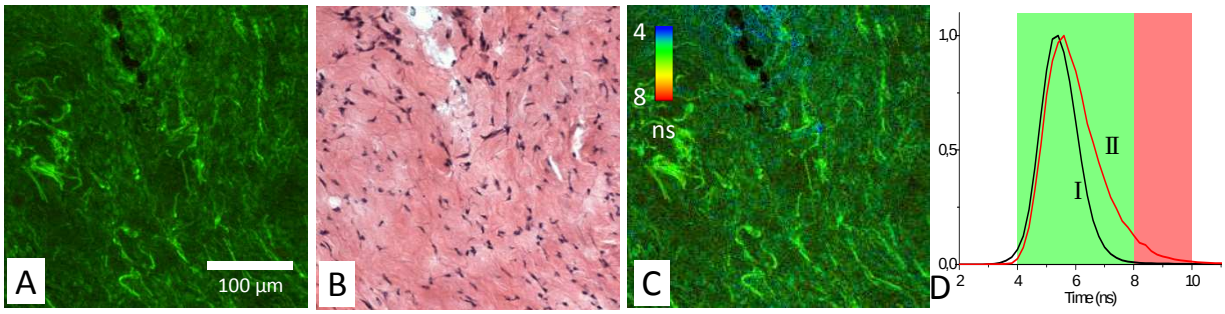


Fig. 16. Images of the healthy synovium specimen: A – a fluorescence image taken combining G and R channels; B – H&E stained histological image; C – a fast FLIM image in **650/160** nm spectral range, total magnification 40x; D – normalized distribution of average fluorescence lifetimes of healthy (I) and sensitized (II) synovium in **650/160** nm spectral range, time gates: green – 4-8 ns, red – 8-10 ns

Fluorescence, FLIM and histological images of the synovium specimen of healthy rabbit are shown in Fig. 16. The green tissue autofluorescence was dominant while the red fluorescence was not distinguished in the combined fluorescence image (Fig. 16, A). Dark spots seen throughout the histological image mark discrete synoviocytes, and the light pink area represents fibrous elements of synovial tissues (Fig. 16, B). The specimen was described morphologically as healthy synovium. The characteristic average lifetimes of fluorophores in the fast FLIM image (Fig. 16, C) of the healthy synovium were distributed from 4 ns to 8 ns (Fig. 16 D). The gating procedure of the FLIM image did not allow distinguishing between cells and fibrous elements of healthy synovium.

Sensitized synovium

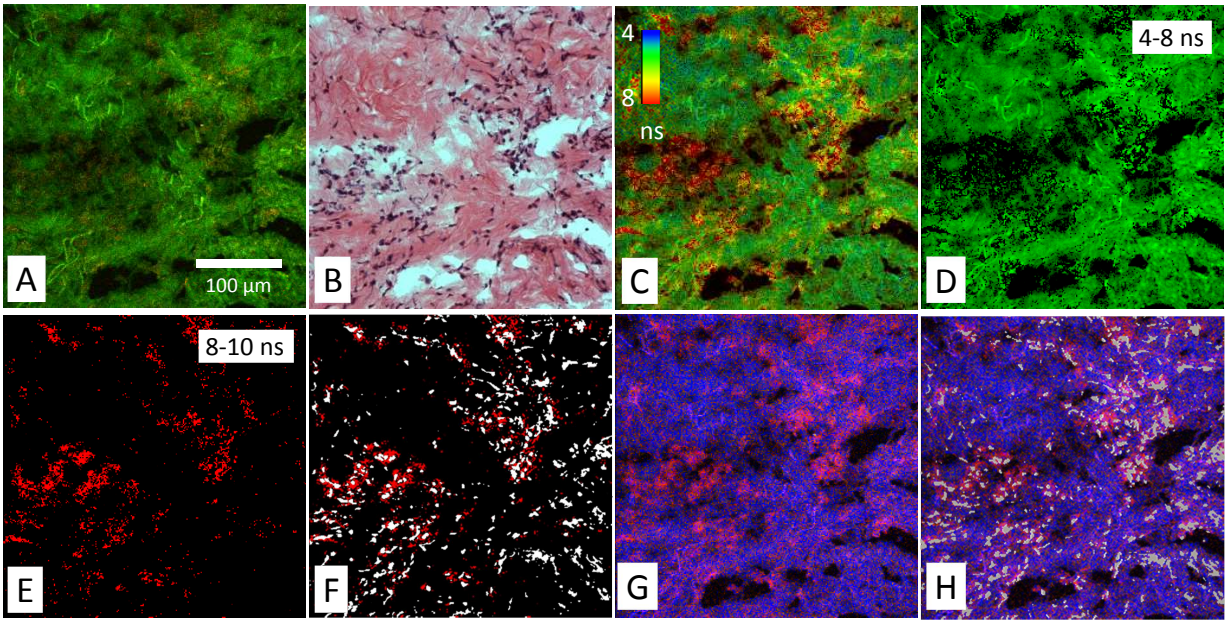


Fig. 17. Images of the sensitized synovium specimen: A – fluorescence image taken combining G and R channels; B – H&E stained histological image; C – a fluorescence lifetime image in **650/160** nm spectral range; D, E – time-gated fluorescence lifetime images (lifetimes gates shown on pictures); F – time-gated fluorescence lifetime image (E) interlaced with a pattern of cells from histological image; G – FLIM image: green colour – $\tau 1$ = 1,3 ns (14 %), blue colour – $\tau 2$ = 4.1 ns (44 %), red colour – $\tau 3$ = 15.4 ns (42 %); H – a FLIM image (g) interlaced with cell pattern from histological image. Total magnification 40x

The characteristic greenish autofluorescence was also dominant over dim red fluorescence in the sensitized synovium specimen (Fig. 17, A). The signs of tissue inflammation in the histological image showed itself as the activation of synovial fibroblasts and the accumulation of inflammatory cells, which were picked out by dark circular area of nuclei (Fig. 17, B). However, the fast FLIM image of sensitized inflamed synovium (Fig. 17, C) differed from that of healthy synovium by presence of fluorescence pattern with longer lifetimes. Average fluorescence lifetimes of fluorophores in the sensitized synovium were prolonged (Fig. 16, D) in comparison with lifetimes of healthy synovium and were found to be limited between 4 ns and 10 ns. The gating of fluorescence lifetime images (Fig. 17, D, E) revealed that the pattern of fibrous elements (Fig. 17, D) can be distinguished at the same average lifetimes as in the healthy synovium – between 4 - 8 ns. The longest average fluorescence lifetimes – 8 - 10 ns (Fig. 17, E) were measured in the cells of the sensitized synovium, and this gated pattern corresponds very well with the pattern of cells (Fig. 17, F), which was retrieved from the

histological image. As it is seen in the calculated FLIM image of the sensitized synovium, the fluorophores with the different fluorescence lifetimes were localized in different sites of the specimen (Fig. 17, G). The inputs of magnitudes calculated for τ_2 (4.1 ns) and τ_3 (15.4 ns) were almost the same in this specimen in the 650/160 nm spectral range. In the FLIM image interlaced with a pattern of cells from the histological image the longest fluorescence lifetime τ_3 (15,4 ns) corresponded to intracellular localization of PpIX, because it was absent in healthy synovium (Table 1). All calculated fluorescence lifetimes for specimens of synovium and cartilage tissue (healthy and sensitized) are presented in Table 1.

Table 1. Fluorescence lifetimes of fluorophores of synovium and cartilage tissues fluorescing in 650/160 nm and 637/13 nm spectral ranges calculated from FLIM measurements. Data expressed as mean ± SD (a number of samples n = 10).

Tissue	Fluorescence lifetime (ns)		
	τ_1	τ_2	τ_3
Healthy cartilage (650/160 nm)	0,75±0,1	3,1±0,2	9,7±0,5
Sensitized cartilage (637/13 nm)	0,8±0,3	3,0±0,1	15,7±0,3
Sensitized cartilage (650/160 nm)	1,2±0,2	4,3±0,3	15,6±0,7
Healthy synovium (650/160 nm)	0,8±0,1	2,9±0,2	8,9±0,2
Sensitized synovium (637/13 nm)	0,9±0,2	2,8±0,3	15,4±0,6
Sensitized synovium (650/160 nm)	1,2±0,1	4,2±0,4	15,2±0,9

3.4. Disscusion

Since there is no ideal minimally invasive method for ablating inflamed synovium in joints, particularly in the smaller joints, of patients with RA, there are numerous attempts to employ endogenous porphyrins for diagnostic and therapeutical purposes in rheumatology as well[21, 23, 24, 27, 28, 40-43]. In order to apply PDT on the inflamed synovium making it safe and suitable approach for the treatment of inflammation in joints, it is essential to prevent damage to the cartilage tissue.

Spectroscopic measurements of synoviocytes revealed that the dominant endogenous porphyrin after incubation with ALA or ALA-Me is PpIX. Average fluorescence intensity values of PpIX in cells from both (RA and OA) groups of patients were insignificantly higher after incubation with ALA in comparison with ALA-Me at almost

all time points. Similar results were obtained in [22], reporting no differences in PpIX accumulation after treatment with ALA or ALA-Me in cells of patients with RA or OA. However, in contrast to [22], we did not obtain significant differences in PpIX accumulation between the larger groups of RA (n_p = 6) and OA (n_p = 6) patients. The detected similar accumulation of PpIX could be caused by absence of native microenvironment characteristic for inflammation *in vivo*. For instance the more active inflammation in the case of RA can have a higher stimulative effect on synoviocytes in comparison with OA [44, 45]. However, biopsy of OA synovium often shows active inflammation, whose signs are close to RA[46]. So, it is likely that PDT can be considered for application to reduce inflammation in the case of OA too.

This study clearly indicates that cartilage explants (Fig. 5) and chondrons in suspensions (Fig. 6) from patients with OA can accumulate PpIX after incubation with ALA, however, at significantly lower levels than synoviocytes. The accumulation of PpIX in CE (Fig. 5, B) and chondrons (Fig. 6, A) was significantly lower at every time point of observation in comparison with synoviocytes (Fig. 4). Moreover, the average fluorescence intensity in CE and chondrons was about ten times lower than in synoviocytes at initial points of measurements. Such disproportion between the fluorescence intensities of PpIX in CE or chondrons and that in synoviocytes can be a result of about six times lower mitochondria content in chondrocytes, which possess metabolism adapted to the hypoxic environment *in vivo*[47, 48] with oxygen concentrations between 1 % and 7 %[49]. These results demonstrate a clear perspective for the selective synovial sensitization with EP, as compared with cartilage, since photodamage occurs in the vicinity of sensitizer[50] and oxygen[51].

Since various HA preparations are applied to the treatment of RA and OA and composition of hyaluronic acid becomes altered during inflammation, chondrons of patients with OA were incubated with 1 mM ALA and HA at the same time to find out whether the application of HA can be related to the accumulation of endogenous porphyrins in chondrons after incubation with ALA. Spectroscopic data of chondrons measured after 12 hours incubation with ALA and HA indicated that PpIX induction was significantly ($p = 0.031$) enhanced in comparison with chondrons incubated only with ALA (Fig. 6, B). The greater accumulation of PpIX inside the chondrons in the presence of HA cannot be explained only by spectroscopic data, therefore, more extensive *in vitro*

and *in vivo* studies are required to investigate the interaction between ALA or its derivatives and a hyaluronic acid, which could also be important in other PDT application areas.

Since the results obtained *in vitro* often are not identical or even contradictory to results obtained *in vivo*, capacity of ALA and ALA-Me to induce PpIX synthesis in the case of RA was studied in the rabbits antigen induced monoathritis model. *In vivo* examination of the fluorescence spectra registered on the skin of the inflamed joints revealed that, regardless of the route of ALA or ALA-Me injection, the dominant endogenous porphyrin in these spectra was PpIX with the fluorescence peak at 635 nm (Fig. 7).

The dependence of the observed fluorescence intensity of exogenously induced PpIX on the route of ALA injection revealed that the maximal value of the averaged intensity on the skin surface of the inflamed joints was about 5 times higher after the i.a. injection than after i.v. injection, whereas the maximal averaged values of PpIX fluorescence on the control joints were not significantly different in these cases (Figs. 8 and 9, A). The contrast between the fluorescence intensities of PpIX on the inflamed and control joints disappeared at two hours after i.v. injection of ALA (Fig. 8, A). The spectroscopic data showed that, from the diagnostic point of view, either lower concentrations of ALA should be used or the fluorescence measurements should be made not later than one hour after application because of diminishing fluorescence contrast between the arthritic and control joints.

When ALA-Me was injected i.a. instead of ALA (Fig. 7), the highest mean value of PpIX fluorescence intensity induced by ALA-Me on the surface of the inflamed joints was about 8 times lower than in the case of ALA (Figs. 7 and 8). ALA induced a higher systemic effect, as indicated by the higher PpIX fluorescence recorded on the skin surface in comparison with ALA-Me (Fig. 8), which is an unfavorable feature for a prosensitizer. The greater spreading of the applied ALA could also contribute to the fading contrast of PpIX fluorescence between the inflamed and control joints at two hours after the i.v. injection (Fig. 9).

To find out whether the spectroscopic differences observed *in vivo* after the application of ALA and ALA-Me can be directly related to the accumulation of endogenous porphyrins in tissues of the inflamed and control joints, the specimens of synovium and cartilage tissues have been analysed *ex vivo*. The spectroscopic assessment of

endogenous porphyrins in the inflamed knee joint tissues indicated that after the i.a. injection of ALA the average PpIX fluorescence intensity in the synovium was two times lower than in cartilage (Fig. 10, A, inset; Fig. 11). The comparison between *ex vivo* fluorescence measurements after the i.a. injection of ALA or ALA-Me revealed the opposite accumulation patterns of endogenous PpIX in the inflamed joint tissues (Fig. 11). The fluorescence of PpIX in the synovium of the inflamed knee joint was not only about two times higher than in cartilage in the case of ALA-Me, but also about 5 times higher than after the application of ALA (Fig. 11). No PpIX fluorescence was detected in the synovium and cartilage specimens of the control joint (Fig. 11), implying that the concentration of the prosensitizers in the circulation was insufficient to induce sensitization of internal tissues. Thus, *in vivo* and *ex vivo* spectroscopic assessment of endogenous porphyrins accumulated in arthritic rabbit tissues showed that the application of ALA is more appropriate for *in vivo* diagnostics of inflamed tissues due to the higher PpIX fluorescence intensity on the skin surface, while ALA-Me in the case of i.a. injection is more appropriate for the therapeutic applications due to the higher and more selective accumulation of PpIX in the inflamed synovium.

Besides PpIX, the fluorescence of water-soluble porphyrins was detected in cartilage tissues (Fig. 12) but did not appear in synovium. The similar mean fluorescence intensity of the water-soluble porphyrins observed in the inflamed and control cartilages, which seems to be invariant with respect to the applied prosensitizer (ALA or ALA-Me) and the type of injection, confirms that induction of arthritis can cause minor systemic sensitization.

Since, during PDT, a photodamage occurs in the vicinity of a sensitizer[50], it is very important to determine the localization of sensitizers in the tissues. Synovium and cartilage tissues of rabbits with antigen induced monoarthritis and those of healthy rabbits were investigated *ex vivo* after intraarticular ALA-Me application by means of fluorescence spectroscopy, fluorescence intensity and lifetime microscopies. In addition, the observed structural features in tissue specimens were validated by histopathological imaging (H&E staining). Structural features of healthy synovium and cartilage were found to be different, and no specific patterns of cells, which had been detected in cartilage tissue, were distinguished in fluorescence and FLIM images of synovium. The presence of endogenous porphyrins, which were assigned to PpIX according to

fluorescence spectra, was detected in specimens of inflamed synovium and cartilage after application of ALA-Me. The fluorescence intensity signal of endogenous porphyrin was too weak for fluorescence microscopy imaging to distinguish specific structures containing PpIX. However, the FLIM images of sensitized specimens were not only very different from the healthy ones, but also revealed the structural patterns of accumulated PpIX possessing the longest fluorescence lifetimes. These patterns very well corresponded with the intratissual sites of cells being identified from the histological images. Calculated fluorescence lifetimes of PpIX, which are much longer than those of autofluorescence, could be used for detection of malignancies or inflamed sites possessing higher amounts of PpIX. Also, calculation of inputs of magnitudes of different decay times could help making the more robust evaluation of PDT processes *in vivo* and real-time dosimetry. Application of lifetime-gating technique allowed to distinguish tissue structures, which were identified from the histological image, by specific average lifetimes, also confirming the supremacy of FLIM imaging over fluorescence microscopy for detection of endogenous porphyrins in sensitized synovium and cartilage specimens.

4. CONCLUSIONS

1. Spectroscopic measurements of endogenous porphyrins revealed that synoviocytes originated from patients with RA or OA possess about ten times higher PpIX fluorescence intensity after incubation with 1 mM ALA or ALA-Me than CE and chondrons of patients with OA. No significant differences of PpIX accumulation were found between RA and OA synoviocytes *in vitro*.
2. Fluorescence intensity of PpIX in chondrons incubated with ALA and the sodium hyaluronate preparation was significantly higher than in chondrons incubated only with ALA.
3. The highest PpIX fluorescence intensity *in vivo* registered on the skin surface of the inflamed knee joints of rabbits with AIA 2 hours after intraarticular injection of either 16 mM ALA or ALA-Me, was up to 8 times higher in the case of ALA. After intravenous 16 mM ALA injection PpIX fluorescence intensity on the skin surface of the inflamed knee joint was about 5 times lower than after intraarticular injection, and almost undetectable after 16 mM ALA-Me injection.
4. PpIX fluorescence intensity in the synovium of the inflamed knee joint *ex vivo* after intraarticular injection of 16 mM ALA-Me was about 5 times higher than in the case of ALA and about two times exceeded the intensity registered in cartilage. The cartilages of both the inflamed and control knee joints of rabbits with antigen induced arthritis accumulate water-soluble porphyrins.
5. Comparison of fluorescence spectroscopy, fluorescence intensity and lifetime microscopy results with the data of histological imaging revealed intracellular localization of PpIX in synovium and cartilage tissues 3 hours after the intraarticular injection of 16 mM ALA-Me.

5. SANTRAUKA (Summary in Lithuanian)

Fluorescencijos matavimas yra vienas iš plačiausiai taikomų būdų optiniam įvairių molekulių kontrastui gauti naudojant skirtingas spektroskopines, mikroskopines ar endoskopines sistemas. Fluorescencija suteikia informacijos ne tik apie tam tikrą fluoroforą bandinyje, bet ir apie fluorescuojančios molekulės ar fluoroforo mikroaplinką. Skirtingi fluoroforai gali būti charakterizuojami remiantis jų žadinimo ir emisijos spektrais, jų kvantiniu našumu, poliarizacija bei fluorescencijos gyvavimo trukmėmis[1-4]. Medicininiuose taikymuose savitoji fluorescencija yra naudojama biologinių audinių ir ląstelių fluoroforų savybių tyrimams. Fluoroforų savybių pokyčiai suteikia informacijos apie biocheminius, funkcinius ar struktūrinius fluoroforų pokyčius audiniuose, kurie atsiranda dėl biocheminių, fiziologinių, metabolinių ar morfologinių audinių pokyčių[1-4, 6-9, 12, 52]. Savitosios fluorescencijos detekcija paremtos technologijos audinių charakterizavimui yra labai tinkamos klinikiniuose tyrimuose, nes papildomai nereikia jokių kontrastinių medžiagų, jos yra minimaliai invazinės, tačiau itin jautrios. Kiekybinė ir kokybinė informacija apie biologinių audinių sudėtį ir patofiziologiją gaunama naudojant nuostoviosios ar kinetinės spektroskopijos metodus[5]. Nuostoviosios fluorescencinės spektroskopijos sistemos yra nesudėtingos ir pigios, lyginant su kitomis, todėl dažniausiai jos yra taikomos diagnostikai *in vivo*. Alternatyvus audinių fluorescencijos detekcijos metodas, kuris nėra jautrus nuostoviosios spektroskopijos signalus įtakojantiems veiksniams, yra laikinės skyros spektroskopija. Fluorescencijos gyvavimo trukmės gali padėti atskirti endogeninius biologinių audinių fluoroforus net jeigu jų fluorescencijos spektrai yra platūs ir persidengiantys.

Kadangi fluorescencinės spektroskopijos matavimo rezultatų negalima susieti su biologinių audinių morfologine struktūra, tam naudojama mikroskopinė audinių analizė, kuri vis dar išlieka auksiniu standartu atpažįstant audinių tipus ar nustatant diagnozę. Biologinių audinių vaizdinimui gerinti, šalia įprastos fazinio kontrasto mikroskopijos, labai susidomėta fluorescencinės mikroskopijos taikymu, ypač po to, kai buvo sukurti konfokalinės ir dvifotonės lazerinės skenuojančios mikroskopijos principai[14, 15]. Fluorescencinės mikroskopijos metodu galima užregistruoti audinių autofluorescenciją ar egzogeninių dažiklių fluorescenciją, tačiau atskirų endogeninių fluoroforų fluorescenciją yra sunku identifikuoti dėl persidengiančių emisijos spektrų. Šią problemą

gali išspręsti fluorescencijos gyvavimo trukmių mikroskopija (FLIM), kurios metu kiekviename vaizdo pikselyje atvaizduojamos būdingos endogeninių fluoroforų gyvavimo trukmės[3, 5, 6, 16, 53]. Taip pat FLIM tyrinėjamos ir FDT naudojamų sensibilizatorių savybės ir jų pokyčiai[8, 17-20]. Šie tyrimai yra labai svarbūs norint tiksliai realiu laiku įvertinti PDT eigą ir prognozuoti gydymo rezultatą. FDT buvo susidomėta ieškant naujų reumatoidinio artrito gydymo būdų[21-27]. Kadangi uždegiminei reumatoidinei membranai būdinga daug požymių, kuriais pasižymi neoplaziniai audiniai, t. y. hipermetabolinis aktyvumas, aktyvi vaskuliarizacija, tikėtina, kad fotodinaminę terapiją būtų galima taikyti ir reumatoidiniam artritui. Fotosensibilizuotos terapijos taikymo principas gydant artritą būtų selektyvi sinovijos destrukcija, kurią sukelia aktyvių deguonies radikalų, susidariusių po fotosensibilizatorių apšvitinimo tam tikro bangos ilgio šviesa, inicijuotos citotoksinės reakcijos uždegiminėse ląstelėse. Į organizmą galima įvesti egzogeninius fotosensibilizatorius arba jų pirmtakus. FDT toks pirmtakas gali būti 5-aminolevulininė rūgštis arba jos dariniai, kurie hemo biosintezės ciklo metu yra paverčiami į endogeninį fotosensibilizatorių protoporfiriną IX. Taip pat šio ciklo metu susidaro ir kiti porfirinai. Kadangi FDT metu fotopažaidos yra sukeliamos artimoje fotosensibilizatorių aplinkoje, labai svarbu tiksliai nustatyti jų sudėtį ir pasiskirstymą audiniuose. Detektuoti endogeninius porfirinus galima spektroskopijos arba mikroskopijos metodais, tačiau norint gauti maksimalų skirtingų fluoroforų kontrastą diagnostikos ar kitais tikslais, neužtenka pasikliauti tik viena spektrine ar gyvavimo trukmių dimensija, kai galima atlikti detalesnę to paties bandinio analizę apjungus kelis spektroskopijos ir mikroskopijos metodus.

Aktualumas

Daugiau nei 100 mln. europiečių serga bent viena reumatine liga. Jos pagal sukeltą ilgalaikį neįgalumą užima 1-ą arba 2-ą vietą iš visų ligų, o išlaidos šioms ligoms gydyti sudaro 25 % visų išlaidų. Reumatoidiniu artritu (RA) pasaulyje serga 0,5-1 % suaugusių žmonių, tai viena iš dažniausių ir svarbiausių reumatinių ligų. Ligos paplitimo dažnumas išsivysčiusiose šalyse yra nuo 5 iki 50 atvejų 100000 žmonių[54]. Be to, šis skaičius kasmet didėja. Geografiškai RA labiausiai paplitęs Šiaurės Amerikoje ir šiaurinėje Europos dalyje, lyginant su kita besivystančio pasaulio dalimi[55]. Taigi, ši liga yra labai aktuali ir Lietuvoje. Visuomenės sveikatos stebėsenos fondo skelbiamo epidemiologinio

tyrimo duomenimis Lietuvoje 2013 metais RA sirgo 12081 pacientų, iš kurių net 80 % yra moterys. Nors atrodytų, kad RA nesukelia tiesioginio pavojaus gyvybei, vis dėlto ligonių sergančių šia liga vidutinė gyvenimo trukmė yra 3–15 metų trumpesnė negu apskritai jų kartos populiacijos[56]. Dėl sudėtingos RA diagnostikos ir gydymo, RA užimą pirmą vietą tarp visų lėtinių ligų pagal tai, kokia dalis pacientų tampa neįgaliaisiais; per 10 metų tai nutinka net apie 50 % pacientų. Kaip ir visų kitų ligų, ankstyva diagnostika yra vienas svarbiausių sėkmingo RA gydymo etapų, tačiau RA ankstyva diagnostika yra labai sudėtinga. Diagnozavus ligą, RA gydymui gali būti skiriamas medikamentinis gydymas nesteroidiniais vaistais nuo uždegimo, steroidiniais hormonais, klasikiniais patogeninės terapijos vaistais ar biologinės terapijos preparatais[29]. Nors yra daug pasiekta RA konservatyvaus gydymo srityje, ypač taikant biologinę terapiją, daugumai ligonių anksčiau ar vėliau prireikia ir chirurginės pagalbos. Taigi vis dar yra daug neišspręstų problemų RA diagnostikoje ir terapijoje, kurios skatina inovatyvių metodų paiešką. Gydant RA norima pasiekti ilgalaikį, slopinantį uždegiminius procesus poveikį atliekant trumpus intensyvius terapinius kursus, kurie sukeltų ligos remisiją. Šiam tikslui pasiekti reikia naujų vaistų, biologinių žymenų ir technologijų, leidžiančių tiksliai įvertinti ligos stadiją ir paciento būklę.

Naujumas

Šiuose tyrimuose pirmą kartą reumatoidinio artrito atveju norint kuo selektyviaus sukelti protoporfirino IX sintezės indukciją uždegiminėje sinovijoje buvo panaudotas 5-aminolevulininės rūgšties metilo esteris, kuris jau yra aprobuotas aktininei keratozei ir pamatinių ląstelių karcinomai gydyti.

Norint išsiaiškinti ar ligos pažeisti kremzlės audiniai geba indukuoti endogeninius porfirinus juos paveikus ALA ar jos dariniais, pirmą kartą pademonstruotos endogeninių porfirinų kaupimosi kinetikos osteoartritu sergančių pacientų kremzlės bandiniuose *ex vivo* ir chondronuose *in vitro* po inkubacijos su ALA ar ALA-Me. Taip pat pirmą kartą tirta hialurono rūgšties preparato įtaka endogeninių porfirinų indukcijai chondronuose, kurie buvo inkubuojami kartu su natrio hialuronatu ir ALA.

Remiantis fluorescencinės spektroskopijos duomenimis palygintos pirmtakų ALA ir ALA-Me savybės indukuoti endogeninius porfirinus po intraveninės ar intrasąnarinės injekcijos antigenu sukeltame triušių monoartrito modelyje *in vivo* ir audiniuose *ex vivo*.

Iš fluorescencijos spektrų pokyčių nustatyta endogeninių porfirinų sudėtis ir jų kiekiai sinovijos ir kremzlės audiniuose.

Pirmą kartą atlikti endogeniniais porfirinais sensibilizuotų audinių fluorescencinės gyvavimo trukmių mikroskopijos tyrimai. Taip pat atlikti triušio kelio sąnario kremzlės pjūviai jos nedekalcinavus. Pirmą kartą atlikti tos pačios bandinio vietos fluorescencinės spektroskopijos bei intensyvumo ir gyvavimo trukmių mikroskopijos tyrimai ir vėliau sulyginti su histologiniu vaizdu. Ši metodika įgalina nustatyti endogeninių porfirinų sudėtį ir jų lokalizaciją biologinių audinių bandiniuose.

Disertacijos tikslas:

Spektroskopiniais ir mikroskopiniais metodais ištirti endogeninių porfirinų susikaupimą ir pasiskirstymą eksperimentinio reumatoidinio artrito modelyje ir pacientų pooperacinėje medžiagoje po 5-aminolevulininės rūgšties ar jos metilo esterio panaudojimo.

Disertacijos uždaviniai:

1. Palyginti endogeninių porfirinų kaupimąsi reumatoidiniu artritu ir osteoartritu sergančių pacientų sinoviocituose, chondronuose ir kremzlės bandiniuose inkubavus juos terpėse su ALA ar ALA-Me.
2. Ištirti hialurono rūgšties preparato įtaką endogeninių porfirinų indukcijai chondronuose.
3. Palyginti ALA ir ALA-Me indukuotų endogeninių porfirinų kaupimąsi triušių eksperimentinio AIA modelyje *in vivo* intraveninio ir intrasąnarinio preparatų suleidimo atvejais.
4. Įvertinti endogeninių porfirinų susikaupimą AIA triušių sinovijos ir kremzlės audiniuose *ex vivo* po ALA ar ALA-Me injekcijos.
5. Nustatyti endogeninių porfirinų lokalizaciją sensibilizuotuose sinovijos ir kremzlės bandiniuose.

Ginamieji teiginiai:

1. RA ar OA sinoviocitai 24 valandų laikotarpiu sukaupia nuo 5 iki 10 kartų daugiau PpIX negu kremzlės bandiniai ir chondronai po inkubacijos su ALA ar ALA-Me. RA ir OA sinoviocitai sukaupia tokius pat PpIX kiekius po inkubacijos su šiais pirmtakais.

2. ALA ir ALA-Me vienodai veiksmingai skatina PpIX susikaupimą bandiniuose *in vitro* bei *ex vivo*.
3. Hialurono rūgšties preparatai skatina PpIX kaupimąsi chondronuose *in vitro*.
4. Triušių eksperimentiniame AIA modelyje *in vivo* PpIX fluorescencijos intensyvumas uždegiminio kelio odos paviršiuje yra apie 8 kartus didesnis po intrasąnarinės 16 mM ALA injekcijos negu po ALA-Me. O didžiausias intensyvumas registruojamas praėjus 2 valandoms po šių pirmtakų injekcijos.
5. PpIX fluorescencijos intensyvumas po intraveninės 16 mM ALA injekcijos uždegiminio kelio odos paviršiuje sumažėja apie 5 kartus lyginant su intrasąnarine injekcija, o po intraveninės 16 mM ALA-Me injekcijos – praktiškai neregistruojamas.
6. Vertinant indukuoto PpIX fluorescencijos intensyvumą uždegiminėje sinovijoje intrasąnarinė ALA-Me injekcija yra apie 5 kartus veiksmingesnė negu ALA. Triušių, kuriems antigenu sukeltas monoartritas, uždegiminio ir kontrolinio kelio sąnario kremzlėse kaupiasi vandenyje tirpūs porfirinai.
7. Praėjus 3 valandoms po intrasąnarinės ALA-Me injekcijos *in vivo*, PpIX lokalizuojasi sinovijos ir kremzlės audinių ląstelėse.

Pagrindiniai rezultatai

Šie tyrimai atlikti tikintis ateityje endogeniniais porfirinais pagrįstą sensibilizaciją pritaikyti diagnostikos ir terapijos tikslais reumatoidinio artrito atveju. Kad fotodinaminė terapija būtų saugus ir tinkamas metodas slopinti sinovijos uždegimą sąnariuose, pirmiausia reikia įsitikinti, ar nebus sukelti kremzlės pažeidimai. Todėl yra svarbi optimalių ALA ar jos darinių panaudojimo sąlygų, tinkančių FDT ar FD, paieška, siekiant padidinti endogeninių pofirinų kaupimosi sinovijoje selektyvumą ir kartu apsaugoti kremzlines struktūras nuo fotosensibilizuotų pažaidų bei sumažinti sisteminį endogeninių porfirinų poveikį organizmui. Šiame darbe buvo atlikti endogeninių porfirinų detekcijos tyrimai *in vitro, ex vivo* ir *in vivo* pacientų pooperacinėje medžiagoje ir triušių antigenu indukuoto monoartrito modelyje.

Atlikus tyrimus su pacientų pooperacine medžiaga, buvo nustatyta, kad vidutinis PpIX fluorescencijos intensyvumas RA ar OA sinoviocituose beveik visais laiko momentais buvo nereikšmingai didesnis po inkubacijos su ALA negu su ALA-Me (4 pav). Šie

pirmtakai taip pat indukavo vienodus PpIX kiekius ir OA kremzlės bandiniuose. Taigi, ląstelėse *in vitro* ar kremzlės bandiniuose *ex vivo* šie pirmtakai vienodai veiksmingai indukuoja PpIX sintezę.

Spektroskopiškai nenustatytas PpIX kaupimosi skirtumas RA ar OA sergančių pacientų sinoviocituose *in vitro* po inkubacijos su ALA ar ALA-Me gali būti dėl savitos mikroaplinkos, būdingos sinoviocitams *in vivo* tam tikros ligos atveju, nebuvimo. Tačiau abiejose ląstelių grupėse buvo aptikta žymių PpIX kaupimosi skirtumų tarp individualių pacientų ląstelių populiacijų. Manoma, kad aktyvesnis uždegimas RA atveju gali labiau skatinti sinoviocitų metabolizmą negu OA atveju ir, tikėtina, kaupti didesnius PpIX kiekius[44, 45]. Kita vertus, atlikta tyrimų parodžiučių, kad ir OA sergančių pacientų sąnario dangalo biopsijai dažnai būdingi uždegiminiai pokyčiai[46]. Taigi, tikėtina, kad RA ir OA pacientų grupėse galėjo būti RA pacientų su silpnesniu uždegimu ir OA pacientų su aktyvesniu uždegimu. Todėl, negalima atmesti prielaidos, kad FDT galėtų būti pritaikyta uždegimui mažinti ir OA ligos atveju.

Svarbiausias tyrimų su pacientų pooperacine medžiaga rezultatas yra tai, kad OA sergančių pacientų kremzlės bandiniai (5 pav.) ir chondronai (6 pav.) po inkubacijos su PpIX pirmtakais 24 valandų laikotarpiu sukaupia nuo 5 iki 10 kartų mažiau PpIX negu RA ar OA pacientų sinoviocitai. Didžiausias PpIX intensyvumų skirtumas yra stebimas pradiniu inkubacijos su pirmtakais laikotarpiu (iki 8 val.). Šis PpIX fluorescencijos intensyvumų skirtumas tarp kremzlės bandinių ar chondronų ir sinoviocitų gali būti nulemtas apie šešis kartus mažesnio mitochondrijų kiekio chondrocituose, kurių metabolizmas prisitaikęs prie hipoksinės aplinkos *in vivo*, kurioje deguonies koncentracija yra nuo 1 iki 7 proc.[47, 49]. Tai patvirtina galimybę selektyviai sensibilizuoti uždegiminę sinoviją ir atliekant FDT išvengti kremzlės pažaidų, nes fotopažaidas daugiausia sukelia aktyvių deguonies formų inicijuotos citotoksinės reakcijos.

Kadangi RA ir OA gydymui yra naudojami įvairūs hialurono rūgšties preparatai bei uždegiminio proceso metu sąnariuose yra pakitusi hialurono rūgšties sudėtis, buvo atlikti tyrimai, siekiant išsiaiškinti, kokią įtaką hialurono rūgšties preparatų panaudojimas galėtų turėti indukuotai PpIX sintezei kremzlės audinyje. Spektroskopiniai duomenys parodė, kad PpIX kiekis chondronuose po 12 val. inkubacijos su ALA ir HA buvo reikšmingai didesnis negu chondronuose inkubuotuose tik su ALA. Atliktų tyrimų duomenimis ALA ir HA sąveikos sąlygotas didesnis PpIX kaupimasis chondronuose

negali būti paaiškintas, todėl reikalingi tolimesni *in vitro* ir *in vivo* įvairių audinių tyrimai šiai ALA ir jos darinių sąveikai su hialurono rūgšties preparatais ištirti, kuri gali būti svarbi ir kitose FDT taikymo srityse, pvz., dermatologijoje.

Kadangi *in vitro* tyrimų metu gauti rezultatai dažnai būna netapatūs ar netgi priešingi *in vivo* gautiems rezultatams, ALA ir ALA-Me savybės indukuoti PpIX sintezę reumatoidinio artrito atveju buvo tiriamos triušių antigeno indukuoto monoartrito modelyje. Uždegiminių ir kontrolinių sąnarių spektroskopiniai tyrimai *in vivo*, matuojant fluorescencijos spektrus odos paviršiuje, atskleidė, kad vidutinis uždegiminių sąnarių savitosios fluorescencijos spektro intensyvumas srityje nuo 500 nm iki 650 nm yra mažesnis negu kontrolinių kelio sąnarių. Tačiau dėl daugybės veiksnių, darančių įtaką absoliutaus fluorescencijos intensyvumo matavimams audiniuose, šis užregistruotas skirtumas yra per mažas, kad juo remiantis būtų galima diagnozuoti uždegimines vietas.

Atlikus sensibilizuotų audinių fluorescencijos matavimus *in vivo*, nustatyta, kad nepaisant to, koks ALA injekcijos būdas buvo naudotas, endogeninių porfirinų spektruose dominavo PpIX fluorescencija, tačiau jo fluorescencijos intensyvumas priklauso nuo pirmtako injekcijos būdo.

Po i.a. ALA injekcijos PpIX fluorescencijos intensyvumai uždegiminiame ir kontroliniame sąnariuose *in vivo* matuojant odos paviršiuje buvo apie aštuonis kartus didesni negu po i.a. ALA-Me injekcijos. Didžiausias PpIX fluorescencijos intensyvumas uždegiminiame ir kontroliniame sąnariuose buvo užregistruotas praėjus 2 valandoms po i.a. pirmtakų injekcijos. Didesnis PpIX fluorescencijos intensyvumas odos paviršiuje po ALA injekcijos negu po ALA-Me reiškia, kad ALA sukelia didesnį sisteminį efektą – bendrą audinių sensibilizaciją, kuri yra nepageidautina.

Po i.v. ALA injekcijos patikimas kontrastas buvo tik pirmą valandą po suleidimo, o vėliau PpIX fluorescencijos intensyvumas buvo beveik vienodas uždegiminiame ir kontroliniame sąnariuose (9 pav., A), tačiau maksimalus intensyvumas uždegiminiame sąnaryje buvo apie penkis kartus mažesnis negu po i.a. ALA injekcijos. Taigi, remiantis šiais rezultatais, kad išliktų patikimas kontrastas tarp uždegiminių ir kontrolinių audinių, diagnostikos tikslais reikėtų naudoti mažesnes ALA dozes arba spektroskopinius matavimus atlikti ne vėliau kaip 1 valandą po i.v. pirmtako injekcijos.

Siekiant sužinoti, ar endogeninių porfirinų fluorescencijos skirtumai, stebėti *in vivo* po ALA ar ALA-Me injekcijos, atspindi uždegiminio ir kontrolinio sąnario vidiniuose

audiniuose susikaupusių porfirinų kiekį, buvo atlikti sinovijos ir kremzlės audinių spektroskopiniai tyrimai *ex vivo*. Uždegiminių audinių spektroskopiniai tyrimai *ex vivo* pademonstravo priešingus PpIX kaupimosi audiniuose rezultatus po i.a. ALA ar ALA-Me injekcijos negu *in vivo*. Po ALA-Me injekcijos PpIX fluorescencijos intensyvumas uždegiminėje sinovijoje buvo apie 2 kartus didesnis negu kremzlėje ir net apie penkis kartus didesnis negu po ALA injekcijos (11 pav.). Be to, ALA indukavo apie du kartus daugiau PpIX kremzlėje negu uždegiminėje sinovijoje tirtu farmakokinetikos momentu (praėjus 4 val. po injekcijos). Šis rezultatas yra prieštaringas *in vitro* atliktų tyrimų rezultatams, kurių metu ALA OA kremzlės bandiniuose indukavo apie 8 kartus mažiau PpIX negu RA ar OA sinoviocituose. Taigi, FDT tikslais tik ALA-Me, o ne ALA, galėtų būti naudojamas mažinti sinovijos uždegimui.

Kremzlės audiniuose, be PpIX fluorescencijos, buvo aptikta ir vandenyje tirpių porfirinų fluorescencija, kurios nebuvo sinovijoje (10 pav.). Panašus vidutinis vandenyje tirpių porfirinų fluorescencijos intensyvumas išmatuotas tiek uždegiminio sąnario, tiek kontrolinio sąnario kremzlėse, nepaisant, koks pirmtakas ar jo suleidimo būdas buvo naudotas, patvirtina, kad artrito sukėlimas sąlygoja silpną viso organizmo sensibilizaciją (12 pav.). Tačiau šių porfirinų buvimas kremzlės audinyje nesukelia problemų FDT metu, nes jų kiekiai, palyginus su PpIX, yra maži ir jie greičiausiai lokalizavęsi ne gyvybiškai svarbiose ląstelės srityse.

Kadangi FDT atveju labai svarbi yra sensibilizatorių lokalizacija, nes fotopažaidos sukeliamos tik fotovaisto aplinkoje, reikia tiksliai nustatyti, kur audinyje yra sukaupęs sensibilizatorius. Remiantis spektroskopiniais tyrimais buvo nustatyta, kad sensibilizuotame triušių kremzlės audinyje yra ne tik PpIX, bet ir vandenyje tirpių porfirinų, kurių fluorescencijos spektrai yra persidengę su PpIX spektru. Atlikti fluorescencinės spektroskopijos tyrimai neįgalina nustatyti fluoroforų pasiskirstymo audinyje. Todėl triušių sinovijos ir kremzlės audinių pjūviuose endogeninių porfirinų detekcija buvo atlikta fluorescencijos intensyvumo ir gyvavimo trukmių mikroskopijos metodikomis, kurių rezultatai vėliau sulyginti su HE dažytais histologiniais vaizdais, ir fluorescencinės spektroskopijos rezultatais. Atlikus sensibilizuotų audinių pjūvių fluorescencinės mikroskopijos tyrimus, nustatyta, kad tirtame spektriniame intervale žalia audinių savitoji fluorescencija yra dominuojanti, o raudona endogeninių porfirinų fluorescencija yra labai silpna. Tačiau vidutinių fluorescencijos gyvavimo trukmių FLIM

vaizduose galima nustatyti endogeninių porfirinų lokalizaciją. Be to, FLIM vaizdą išskaidžius į trumpesnius laiko intervalus, galima atskirti skirtingas audinių morfologines struktūras. Sulyginus tų pačių bandinių FLIM ir histologinius vaizdus, nustatyta, kad sensibilizuotoje sinovijoje ir kremzlėje endogeniniai porfirinai yra ląstelėse. Sensibilizuotų sinovijos ir kremzlės bandinių fluorescencinės spektroskopijos tyrimų rezultatai atskleidė, kad endogeninių porfirinų spektruose užregistruotos tik PpIX ir jo fotoprodukto spektrų juostos. Tačiau reikia paminėti, kad bandinių paruošimo metodai galėjo turėti įtakos vandenyje tirpių endogeninių porfirinų neaptikimui bandiniuose. Atlikus detalesnę FLIM vaizdų analizę, buvo apskaičiuotos tikrosios fluoroforų fluorescencijos gyvavimo trukmės. PpIX fluorescencijos gyvavimo trukmė yra daug ilgesnė negu savitąją sveikų audinių fluorescenciją sudarančių fluoroforų, todėl ji galėtų būti panaudota tiksliai navikinių darinių ar uždegiminių sričių, kuriose aptinkami didesni PpIX kiekiai, diagnostikai. Taip pat apskaičiuotos ir fluoroforų fluorescencijos gyvavimo trukmių amplitudės. Jų kitimas, pvz., blunkant PpIX mažėjantis gyvavimo jo trukmės indėlis, galėtų būti panaudoti tobulinant FDT procesų eigos ir dozių vertinimą *in vivo.*
Taigi, suleidus ALA-Me intrasąnarinių būdu, galima sukelti didenę uždegiminės sinovijos sensibilizaciją, negu aplinkinių audinių. Tačiau, norint selektyviai atlikti sinovijos abliaciją FDT, dar reikia tiksliai kiekybiškai įvertinti PpIX kiekį audiniuose ir apkaičiuoti šviesos sklidimą sąnario audiniuose, kad būtų galima tiksliai apskaičiuoti FDT dozę.

Išvados:

1. Atlikus spektroskopinius tyrimus, nustatyta, kad RA ir OA sergančiųjų sinoviocitai po inkubacijos su 1 mM ALA ar ALA-Me sukaupia nuo 5 iki 10 kartų didesnius PpIX kiekius, negu OA sergančiųjų kremzlės bandiniai ir chondronai. Tačiau RA ir OA sinoviocituose po inkubacijos su šiais pirmtakais PpIX kaupimosi skirtumai nenustatyti.
2. PpIX fluorescencijos intensyvumas chondronuose inkubuotuose kartu su ALA ir hialurono rūgšties preparatu reikšmingai didesnis negu inkubuotuose tik su ALA.
3. Didžiausias PpIX fluorescencijos intensyvumas triušių AIA modelio uždegiminio sąnario odos paviršiuje, užregistruotas *in vivo* praėjus 2 valandoms po intrasąnarinių 16 mM ALA ar ALA-Me injekcijų, buvo apie 8 kartus didesnis

ALA atveju. Po intraveninės 16 mM pirmtakų injekcijos PpIX fluorescencijos intensyvumas uždegiminio kelio odos paviršiuje ALA atveju buvo apie 5 kartus mažesnis negu po intrasąnarinės injekcijos, o ALA-Me atveju – praktiškai neregistruojamas.

4. PpIX fluorescencijos intensyvumas uždegiminėje sinovijoje *ex vivo* po intrasąnarinės 16 mM ALA-Me injekcijos buvo apie 5 kartus didesnis negu ALA atveju ir apie 2 kartus viršijo intensyvumą išmatuotą kremzlėje. Triušių, kuriems antigenu sukeltas monoartritas, uždegiminio ir kontrolinio sąnario kremzlės audiniuose kaupiasi ir vandenyje tirpūs porfirinai.
5. Ištyrus triušių sąnarių audinius fluorescencinės spektroskopijos, fluorescencijos intensyvumo ir gyvavimo trukmių mikroskopijos metodais bei gautus vaizdus sulyginus su HE dažytais histologiniais vaizdais nustatyta, kad po intrasąnarinės ALA-Me injekcijos praėjus 3 valandoms PpIX yra susikaupęs uždegiminio sąnario sinovijos ir kremzlės audinių ląstelėse.

6. LIST OF PUBLICATIONS

1. Publications listed in the Thomson Reuters Web of Science database:

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