VILNIUS UNIVERSITY

Edvardas Žurauskas

EVALUATION OF THE HUMAN HEART CONDUCTION SYSTEM VISUALIZATION POSSIBILITIES ACCORDING TO MORPHOSPECTRAL AND PROTEOMIC INVESTIGATIONS

SUMMARY OF DOCTORAL DISSERTATION

Biomedical sciences, Medicine (07 B)

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The study was carried out at the Vilnius university during the period 2001-2009.

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The public defense of the dissertation will be held at the open meeting of the Medical research council of Vilnius university, in the conference hall of the National centre of pathology, at 13 p.m. on 9 April, 2010.

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The summary of the doctoral dissertation has been sent on 9 March 2010.

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VILNIAUS UNIVERSITETAS

Edvardas Žurauskas

ŽMOGAUS ŠIRDIES LAIDŽIOSIOS SISTEMOS VAIZDINIMO GALIMYBIŲ ĮVERTINIMAS PAGAL MORFOSPEKTRINIUS IR PROTEOMINIUS TYRIMUS

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Introduction

Conduction system of the human heart is a specialized myogenic tissue that generates and transmits bioelectrical impulse. This impulse produces synchronical excitation and contraction of the distinct part of the myocardium. Components of the conduction system are found in all parts of the human heart and miscellaneous pathological process can damage this system with the successive heart malfunction. Impulse spreading may discontinue at any part of the conduction system: at the sinus or atrioventricular node, His bundle (HB) or in one or both bundle branches. The conduction and rhythm disturbances in the child‘s heart most often are related to congenital defects and ectopic focuses of the conduction system. Congenital additional conduction bundles (Kent bundle) may evoke Wolff-Parkinson-White syndrome with supraventricular tachycardia and cause sudden death. Diverse heart diseases with rhythm and conduction disarray are the most often source of death in a civilized world. Anatomical, microscopic and biochemical heart analysis cannot explain the cause of death of about 12% of sudden cardiac deaths.

As a separate human heart structure conduction system it was described at the beginning of the XX century, but for about five decades it had been argued whether this system existed as a detached structure of the human heart. Rather complex morphological investigation methods of the human heart conduction system had raised these discussions. It is difficult to identify the conduction system of the human heart on grossly or microscopical examination and demonstrate subjection of the tissue. Human heart conduction system tissues are hardly distinguishable grossly and histologically from the surrounding heart tissues, and to prove on purpose the dependence of the tissue to the conduction system it is essential to establish the relation between the investigative tissue and other parts of the conduction system. For this point it is necessary to investigate all ventricular conduction system, and this job demands proficiency, huge input of time and activity. For the mentioned above investigation difficulty, the knowledge about conduction system of the human heart is bitty and morphological changes poorly correlate with clinical picture. There is no unanimous opinion for the present about the existence of anatomical conduction pathways between the sinus and atrioventricular nodes, scientific literature represents unequal measurements of different

parts of the conduction system. Distribution and incidence of anatomical variants and congenital defects of the human conduction system are unknown. Location of the conduction system remains completely vague in the majority of cases of congenital heart defects. Death, induced by established arrhythmias, may remain inexplicable by morphological study, or apparent pathology may be found in the heart of a patient who has never displayed clinical evidence of abnormal conduction.

This is not the cause for dismay but testifies to the difficulties of such studies and to our incomplete knowledge of cardiac conduction. Studies on anatomical conduction pathways proved to be too complex for the majority of investigators and they are not carried out any more in the present time. Descriptions of this system are based on topographical schemes reconstructed from histological slides. How exactly these schemes correspond to reality it is impossible to check due to destruction of the heart anatomical preparation.

Morphological differences allowing distinguishing conduction system tissue from an ordinary myocardium are described controversially in the literature. Such discrepancies may be explained by different analysis methods and investigation of different parts of the conduction system. For example, the atrioventricular node contains many nervous plexuses with a huge amount of succinate dehydrogenase and cholinesterase, however these enzymes are not found in other parts of the conduction system.

A specific visualization method of the human heart conduction system does not exist. Such method would be very useful for embryological, anatomical examination of the conduction system and for clinical practice. The moveless heart under the knife does not demonstrate electrical activity and it is impossible to locate the conduction system by electrophysiological method. Atypical conduction system position may cause surgical lesion and serious post-operative complications. Visualization of the human heart conduction system may support avoidance of intra-operative damage of this system.

Although anatomy and histology of the human heart conduction system were described more than 100 years ago there is nowadays the necessity to specify and supplement for better knowledge. For this purpose it is necessary to create a specific, secure, unsophisticated and useful visualization method for the workaday examination of the conduction system.

Aim of the study

To identify morphological and spectroscopic differences between the conduction system of the human heart, ordinary myocardium and heart connective tissue; to create the visualization method of the conduction system of the human heart according to these differences.

Objectives

1. To create the preparation method of the conduction system of the human heart, which would enable to receive the conduction system tissue with no other heart tissues and propose tissue samples of the human heart for spectroscopic and proteomic investigations.
2. To examine X-ray electron, infrared and ultraviolet absorption spectra, specific fluorescent emission and fluorescent excitation spectra of the conduction system and other human heart tissues; to estimate parameters and spectral regions where significant differences between tissues are evident.
3. To compare proteomic composition of the conduction system and other human heart tissue homogenates by electrophoresis, to examine spectrical features of the homogenates and register proteomic differences.
4. To summarize investigation results and find statistically reliable differences between the conduction system tissue and other heart tissues; to evaluate possibilities to consider these differences for the creation of the conduction system visualization method.
5. To perform visualization experiments of the human heart conduction system.

Defended statements

1. Ions are very important for the rise of bioelectrical impulse in the conduction system of the heart, but X-ray electron microscopy investigations show that the amount of phosphorus, calcium, chlorine, sulphur, silicon, and sodium and potassium ions is equal in the conduction system and ordinary myocardium.

Therefore, the X-ray electron microscopy method can not be applied to separate the conduction system tissue from the myocardium.

2. Differences between His bundle tissue and the myocardium stated by Fourier transformations (FT-IR) infrared absorption spectroscopy method allows us to conclude that these tissues are morphologically distinct. There is no possibly to explain exact reasons of these differences and to attribute particular FT-IR absorption bands to vibrations of concrete molecular groups because absorption spectra of the biological tissues are whole of the protein amino acids, peptides, and free amino acid spectra, unambiguously do not relate to morphology. However, it is possible to separate the conduction system tissue and myocardium by permeability ratio of the IR spectrum bands approximately at 1450 cm^{-1} and 1400 cm^{-1}.
3. UV absorption spectra of the human heart His bundle and the myocardium are different by intensity at aromatic amino acid absorption region from 250 nm to 300 nm. The analysis shows that His bundle contains 2 times more tryptophan and 4 times more tyrosine than the myocardium. Excitation in this region may cause different fluorescents of the heart tissues and visualization of the conduction system against the heart tissues background.
4. Most prominent differences of the specific fluorescence spectra intensity between His bundle and the myocardium are seen at 420 - 465 nm regions with excitation of 320 - 370 nm.
5. His bundle of the human heart and bundle branches are spectroscopically possible to separate from the connective tissue of the heart by excitation in the absorption bands of the collagen (330 nm) and elastin (385 nm) and by recording variations of the fluorescence intensity at 460 nm.
6. Electrophoresis indicates that the His bundle tissue contain about 26 kDa molecular mass protein group, which was not found in the myocardium and the connective tissue of the human heart.

Scientific novelty and originality

Most of the heart conduction system investigations are performed by using small laboratory animals, the hearts of which are easy to be examined histologically. Research of the human heart conduction system is rarely performed due to technical bothers. There are chiefly described narrow series of the investigated cases with the examination of the particular part of the conduction system. The sinus and atrioventricular nodes are investigated most commonly. These researches are performed using blocs containing human heart tissues with the conduction system fragments. Examination of the isolated human heart conduction system tissues was not performed. Micro/macro preparation method of the conduction system used in our investigation enabled us to get a pure conduction system tissue, without impurity of other heart tissues and to hand over for future investigations. Therefore, performed spectroscopic and proteomic investigations are original, as parallel analysis is not described in the literature.

According to the results of spectroscopic examination of the conduction system and other human heart tissues, for the first time there were estimated optimal circumstances for the separation of these tissues (optimal wavelength for fluorescence excitation and optimal fluorescence registration ranges). There were performed first visualization experiments of the conduction system.

Also there was performed initial comparison proteomic analysis of the conduction system tissue, the connective tissue and the myocardium by using a pure conduction system tissue and there was found a protein group characteristic for conduction system. Original macroscopic and histological pictures, images of the visualization experiments are presented in the dissertation.

Whereas universally accepted morphological differences between the conduction system and other heart tissues are unknown, the investigation performed provides with the new knowledge about characteristic features of the human heart tissues.

Materials and methods

Samples of the human heart conduction system tissues, the myocardium and connective tissue, pancreas tissue (used for comparison) were collected during the

autopsies at the National Centre of Pathology. Then spectral investigation tissue samples were examined by a standard histological technique and the descriptions of the findings were incorporated into the autopsy report. Macroscopical findings noted at a preparation of the heart conduction system were also incorporated into the autopsy report.

Tissue specimens of 77 human hearts were examined. For investigation there were chosen heart preparations of the adults from 20 to 60 years old, without heart pathology or systemic diseases, which could damage the heart. The investigated were chosen irrespective of age, sex, and information about them was not gathered. The heart preparations were numbered from 1 to 77. Tissue specimens were obtained no later than 48 hours after death. The tissues were fixated in the 10% buffer formalin solution, examined immediately or stored at – 70^0C. There were used the tissues of His bundle with its branches and the myocardium for investigation. Pancreas tissues were used for comparison (35 specimens, numbered from K1 to K35) in FT-IR spectroscopic investigations, UV absorption spectroscopic investigation and for histochemical tissue examination.

X-ray electron microscopy

For the X-ray electron microscopy investigations fresh tissues of His bundle (HB) and the myocardium were exsiccated at 80-90^0C (preparations 1-5). Other part of the myocardium and HB tissues were fixated in 10% buffered formalin and exsiccated at 80-90^0C (preparations 6-10). The qualitative elemental structure of the samples was analyzed using the method of SEM-EDX. The spectra were registered with the JOEL JSM-840 scanning electron microscope and LNK analyser AN 10/55S. Circumstances of the analysis: silicon-lithium detector with beryllium window, voltage of the electrons acceleration sound - 20kV, sound current - 3.10^{-9} A, slot - 1.

FT-IR absorption spectroscopy method

For the FT-IR spectroscopic investigations the HB, myocardium and pancreas tissue samples were used fresh (preparations 11-20 and K1-K10), and fixated in 10% buffered formalin and exsiccated at 80-90^{0}C (preparations 21-25 and K11-K15). Spectra were registered in the region of 4000-480 cm^{-1} with the Perkin Elmer FT-IR microspectrometer Paragon 1000PC with the i-series FT-IR microscope.

UV absorption spectroscopy

The His bundle, myocardium and pancreas tissue samples (preparations 26-35 and K16-K25) were fixated in 10% neutral buffered formalin and embedded in paraffin. Sections of 15μm thickness were performed with the Leica RM2145 microtome and placed on quartz slides. Absorption spectra of the samples were recorded applying the PC1000 Plug-in Spectrometer (Ocean Optics, Inc., USA).

For comparison, there were prepared solutions of tyrosine (Tyr) and tryptophan (Trp) 10^{-3} M in distilled water.

Histochemical methods

The His bundle, myocardium and pancreas tissue samples (preparations 36-45 and K26-K35) were fixated in 10% neutral buffered formalin and embedded in paraffin [64]. Sections of 4μm thickness were performed with the Leica RM2145 microtome and stained by Millon reaction for tyrosine, a diazotization-coupling method for tyrosine and a DMAB-nitrite method for tryptophan. The pancreas tissue samples were used as control tissues.

Fluorescent spectroscopy and visualization experiments

The samples of the conduction system tissues used for the spectroscopic studies were abstracted from the site of the HB bifurcation and a proximal part of the left branch by the micropreparation method during the autopsy. The samples for the microscopic visualization experiments (2-3 cm in size) were prepared from the left branch of the HB and taken together with the surrounding tissues of the endomyocardium. The samples prepared from the whole interventricular septum were taken for the macroscopic visualization experiments. Fluorescence excitation and emission spectra of the heart tissue specimens were recorded on a LS 50B spectrofluorimeter (Perkin-Elmer, USA). Since the comparison between the spectra measured from both non-fixed (preparations 46-50) and fixed (preparations 51-55) tissue specimens revealed no significant differences, only specimens fixed in a 10% neutral buffered formalin solution were used for further studies. Every specimen was put between two quartz slides, placed into the sampling chamber of the instrument and fastened to the black background turned at 55° with respect to the excitation beam for the detection of the emission signal from the surface of the specimens. The installed long-pass filters were used optionally to cut off the scattered excitation light. As the fluorescence intensity of tissue samples was low, the values of the slit widths for the excitation and emission monochromators were set at spectral resolutions of 5 nm and 7 nm, respectively.
The presence and location of HB branches in the specimens were investigated by means of the fluorescence microscope Olympus BX 60 under illumination at 366 nm using an UV excitation fluorescence mirror unit U-MWU2 (exc: 330-385 nm, dichr: 400, em: 420 nm) and the images obtained with a digital camera Olympus D 50 were compared with the histological data. Some of the specimens were selected for further fluorescence measurements and the fluorescence spectra were registered by means of the microscope-spectrofluorimeter MC ФУ (Russia) under the same illumination conditions as before using the analogous combination of filters. During the macroscopic visualization experiments (preparation 56-65) the spectral region around 366 nm of the high-pressure mercury lamp ДРШ 250-3 (Russia) was selected by a 5 cm width water filter and a filter УФС 8 (Russia) and Xe lamp

MAX-302 with interference filters were used. The images were taken with the CCD camera ToUcam Pro (Philips) and Infinity 2.

Investigation of the heart tissue extracts

Tissue disruption and preparation of the extracts

Tissues (stored at – 22^{o}C) (preparation 66-75) were weighed, chopped into small (1–5 mm) pieces with a scalpel, washed three times with ice-cold 0.9% KCl, and homogenized within tenfold volume excess of 5 mM Tris homogenization buffer containing 250 mM sucrose and 2 mM EGTA (pH 7.7 at 2°C), using a mini glass Potter homogenizer (0.1–2 ml) with 10–15 strokes. The homogenates were centrifuged for 5 min at 5000 *g*, the supernatant was collected (soluble fraction I) and the pellet was then homogenized within a tenfold volume excess of a buffer which consisted of 5 mM Tris, 180 mM KCl, 8 M urea, 2 mM EGTA (pH 7.7 at 2 °C) as before. The resulting supernatant was collected (soluble fraction II) and the pellet was homogenized within a fivefold volume excess of a buffer containing 50 mM Tris, pH 6.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 5% (w/v) 2-mercaptaethanol and centrifuged again. The resulting supernatant represented the soluble fraction III. The three different extracts from the homogenised heart tissues comprised three subsequent supernatants and the pellet remained as an insoluble fraction after each extraction.

SDS-PAGE

The aliquots of the tissue extracts containing 50 μg of protein were fractionated by sodium dodecyl sulphate – polyacrylamide-gel electrophoresis (10% gels). Gels were stained with Coomassie Blue R-250. Protein concentration was determined by a BCA protein assay.

Spectrophotometric analysis.

Spectrophotometric analysis was performed with the Perkin– Elmer LS-50B spectrometer. Fluorescence and fluorescence excitation spectra of the tissues were recorded *ex vivo* and during various stages of homogenization. Fluorescence reflects the intrinsic features of the tissue which are obtained in the form of fluorescence spectra. Fluorescence excitation spectra at some approximation

represent the absorption of tissue. During fluorescence measurements, the excitation wavelength (λ_{ex}) was kept constant, and the detector scanned the selected wavelength region thus obtaining fluorescence spectra, while measuring fluorescence excitation the detection wavelength (λ_{fl}) was kept constant and the excitation wavelength scanned the selected region

Analyses of liquid samples were performed using a 1 cm thick quartz quvette. Small unstained SDS-gel sections containing an appropriate group of proteins were cut out from gels, fixed on quartz slides and used for spectroscopic investigations in a reflection mode. The spectral excitation and detection slits in all measurements were 5 nm and 7 nm, respectively.

2D gel electrophoresis

Total protein isolation from the heart tissue

Fresh tissue dissected from the human myocardium and heart conduction system (preparation 76-77) was rinsed briefly with an ice-cold buffer (PBS) and blot dry. A tissue sample was pulverized in liquid nitrogen with a pestle until it was a uniform powder. To the pulverized tissue an appropriate volume of the Chemicon Total Protein Extraction Kit reagent was added and the tissue cell lysate was transferred to the tube, and further the heart total proteins were isolated according to the manufacturer's protocol (Chemicon International, Inc.). The total tissue proteins were examined immediately or stored at –70 °C.

Protein fractionation in 2DE system

The total tissue proteins were resolved by two-dimensional gel electrophoresis (2DE). An Immobiline DryStrip Kit, pH range 3–10, and Excel Gel SDS, gradient 8–18% were used for 2DE. The process has been performed according to the manufacturer's instructions (Immobiline DryStrip Kit for 2-D Electrophoresis with Immobiline DryStrip and ExelGel SDS, Amersham Pharmacia, Uppsala, Sweden). For the analysis of the total hart tissue proteins, 2DE gels have been stained with the PageBlue Protein Staining Solution (PageBlue Protein Staining Protocol, Fermentas, Lithuania).

Results

Conduction system of the human heart preparation method

Due to the muscular origin of both the heart conduction system (HCS) and the myocardium (MC) it is complicated to distinguish these two tissues visually. Just the fact that the HCS is surrounded by the connective tissue allowed us to separate it microscopically from the ordinary myocardium. Anatomic atlases can be used as a reference for approximate detection of HCS in the heart, though its precise location, especially in various pathologic cases, still remainsvery problematic.

In the obtained samples the artery of the atrioventricular (AV) node branched from the right coronary artery on the same level as the posterior interventricular sulcus. It was separated up to the middle level of the septal tricuspid leaflet close to the front of the coronary sinus. On this level the artery penetrates the back pole of the AV node or gets into a posterior part of the interventricular septum under the node. From this step of preparation a Carl Zeiss biological stereomicroscope with 12-36 x magnification was used. To find the anterior pole of AV node the connection between a central fibrous body and a fibrous ring of the tricuspid valve had to be disclosed. To perform this, the endocardium, soft tissues and the myocardium had to be separated from the central fibrous body. Going down, the connection between the right slope of the central fibrous body and tricuspid ring was found. It corresponded to the level of the anterior commissure of tricuspid valve. Then the basement of the fibrous ring of the septal tricuspid leaflet was separated from the soft tissues till the back node pole, thus finishing the arterypreparation. In this way the right edge of the node was discovered. The preparation was eased by the fact that the connective tissue coating the AV node was joined to the tricuspid fibrous ring. This allowed precise separation of the right edge of the node. To find the left edge of the node the soft tissues covering AV node were separated 4-6 mm in width (it corresponds to the node width). So the whole AV node was disclosed completely together with the atrial part of His bundle (HB), which is located close to the junction of the right tricuspid fibrous ring with the central fibrous body or 1-1.5 mm behind it. A penetrating part

of the bundle can be easily found after destruction of a junction between the central fibrous body and the fibrous ring of the tricuspid valve. To achieve thefollowing steps were performed: one blade of the scissors was introduced under the right edge of the central fibrous body close to the junction with the tricuspid fibrous ring and the junction was cut. The bifurcation of HB is located on the border between membranous part of the interventricular septum and the crest of the muscular interventricular septum part (Fig. 1).

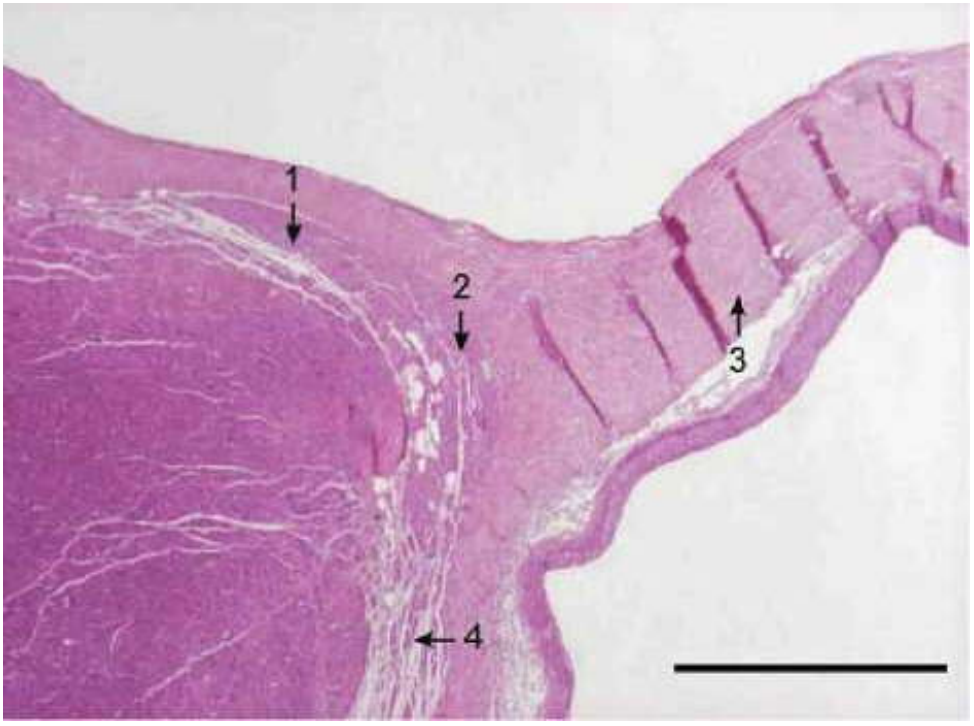

Fig. 1. Section of His bundle (Haematoxylin-Eosin stain, x20). 1 - right branch of HB; 2 - bifurcation of HB; 3 - membranous part of the interventricular septum; 4 - left branch of HB. The scale mark is 1 mm.

Firstly, the top right borders of the HB ventricular part have been separatedfrom the surrounding tissues, and then the left edge (with the very thin left branch) was found. It was not difficult to disclose theHB branches by their fixed location.

X-ray electron microscopy

Evaluation of the qualitative elementary composition of the heart tissues was performed by the X-ray electron microscopy method. Examination results show that the tissues of the myocardium and conduction system contain equal amount of the phosphorus, calcium, chlorine, sulphur, silicon, natrium and potassium ions. Transmission velocity of the bioelectrical impulse probable is unrelated to the different amount of ions in the myocardium and conduction system.

FT-IR absorption spectroscopy method

Analysis of FT-IR absorption spectra show differences between myocardium and conduction system tissues. Differently prepared tissue samples were compared by the FT-IR absorption spectroscopy method. There were no differences found between formalin fixed and dried tissue samples. Investigation of the formalin unfixed and undried His bundle and ventricular myocardium tissue samples show some differences. Formalin fixation and desiccation have no influence on stabile, structural proteins; therefore, differences between diversely prepared tissues possibly are determinate by changes of free cytoplasmic molecules. Such assumptions are indirectly confirmed by finding, that the ventricular myocardium contains more free or minus charged (α-asparagines and α-glutamic) amino acids. What may determine these differences it is difficult to evaluate, because IR absorption spectra of the biological tissue are whole of the protein amino acids and free amino acids spectra. FT-IR method let unambiguously separate the myocardium and conduction system tissues, but a blind spot of this method is complexity of the examination and possibility to examine only small parts of the tissues. This method would be difficult to apply in practice.

UV absorption spectroscopy

Optical spectroscopy usually serves as one of the best noninvasive visualization methods. The basic task for the creation of a working visualization technique would be to determine spectroscopic differences between the conduction system and the myocardium. In the absorption spectra of the conduction system and pancreas tissues, which were chosen for comparison, the absorption band at about 280 nm was detected. In the absorption spectrum of the myocardium tissue the relative intensity of this band is very weak. In addition, significant light scattering was observed in all spectra. The component of the scattered light in the averaged absorption spectra was subsequently removed by the following procedure: in the spectral region of 305-430 nm, where amino acids absorb no light, the spectra were approximated with a class of hyperbolic functions being inversely proportional to the wavelength in the fourth power. The model curves were extrapolated towards shorter wavelengths to fit the spectra, and the obtained corresponding values were subtracted from the initial measured values.

Analysis of the absorption data implies the following presumptions: the absorption of the investigated tissues in is determined by the absorption of amino acid tyrosine (Tyr) and tryptophan (Trp); the His bundle and pancreas tissues contain significantly higher amount of Tyr and Trp than the myocardium tissue.

The use of the absorption spectroscopy in the quantitative of biological objects is based on the statement that optical density of mixture is equal to the sum of optical densities of mixture components. For the rough determination of Trp and Tyr concentrations in the analyzed specimens the following system of equations was solved:

$D_{290} = \varepsilon_{Tyr,290} C_{Tyr}\, l + \varepsilon_{Trp,290} C_{Trp} l;$

$D_{280} = \varepsilon_{Tyr,280} C_{Tyr}\, l + \varepsilon_{Trp,280} C_{Trp}\, l.$

Where D_{290} and D_{280} are optical densities at 290 nm and 280 nm respectively; l is the optical path length, C_{Tyr} and C_{Trp} are the concentrations of Tyr and Trp, ε_{Tyr} and ε_{Trp} are the extinction coefficients of Tyr and Trp at the indicated wavelengths. The values of ε used in the calculation were the following:

$\varepsilon_{Tyr,280}$=1280$M^{-1}cm^{-1}$, $\varepsilon_{Tyr,290}$=333$M^{-1}cm^{-1}$, $\varepsilon_{Trp,280}$=5690$M^{-1}cm^{-1}$, and $\varepsilon_{Trp,290}$=4850$M^{-1}cm^{-1}$. The calculation amounts of Trp and Tyr in the studied tissues are presented in Table 1.

Table 1. Concentration of Trp and Tyr in biological tissues.

Tissue	C_{Tyr}, μM	C_{Trp}, μM
Pancreas	15,0	4,0
Conduction system	21,0	1,5
Myocardium	5,2	0,6

Calculation results show that the concentration of Trp in the conduction system is 2.5 times lower, while concentration of Tyr is 1.4 times higher than in the pancreas tissue. Similar comparison of the conduction system with the myocardium indicated the 2.5 time higher concentration of Trp and the four times higher concentration of Tyr in the His bundle tissue. Based on these semi quantitative evaluations the conclusion can be drawn that specific properties of the conduction system might be determined by the higher concentration of two aromatic amino acids, especially Tyr. Therefore, higher concentration of Tyr and Trp could make it possible to visualize the conduction system of the heart and distinguish it from the myocardium under the excitation in the region of 260-295 nm, where the fluorescence in the conduction system of the heart is expected to be higher than in the myocardium.

Histochemical methods

Histochemical methods were used to prove higher concentration of tryptophan and tyrosine in the human conduction system. Unfortunately all these methods did not show a major amount of Tyr and Trp in the conduction system tissues than in the myocardium. The reasons for this failure remain unclear.

Fluorescent spectroscopy and visualization experiments

The fluorescence measurements on the heart tissue specimens were started under excitation in the spectral range of the absorption of aromatic amino acids (255 nm and 280 nm). An intensive band of Trp fluorescence with a peak at about 340 nm was observed in the spectra measured from all types of heart tissues (Figs. 2 and 3). The fluorescence of Trp, however, could not serve for unambiguous discrimination between the spectra of HB and the myocardium, since despite slight differences in a peak position and bandwidth the shape of this band was found being non-specific to a particular type of tissue. On the other hand, the shape and relative intensity of the fluorescence spectra differed in the spectral range from 400 to 500 nm (Fig. 2), where no fluorescence of Trp residue is expected. The relative fluorescence intensity of HB in this region was higher than that of the myocardium.

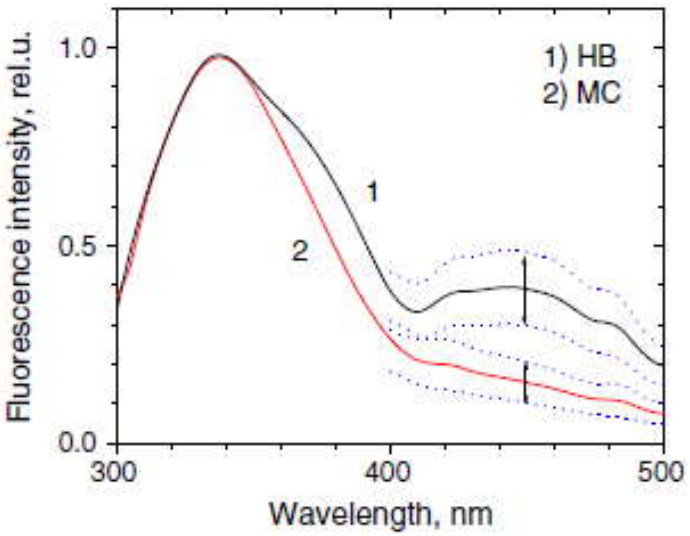

Fig. 2. Averaged fluorescence spectra (excitation at 255 nm) of His bundle and the myocardium tissues normalized at peak values. Dotted lines mark the standard deviation.

Looking for the possibility of the precise differentiation between His bundle and the myocardium tissue, the ratios of the fluorescence intensity at 340 nm to that at 450 nm were calculated from the averaged fluorescence spectra (Fig. 2) for both types of tissues. For HB this ratio was 2.5 ± 0.8 and for the myocardium (MC) 6.4 ±1.8 (a standard deviation was used for error rating, the calculated reliability of differences between the fluorescence intensities of the HB and MC was more than 99 % ($p < 10^{-7}$), when measured at 450 nm).

These results indicated a possibility for a good differentiation between the HB and MC spectra under UV exposure by comparing the ratios of the fluorescence intensities measured at 340 nm to those at 450 nm. Though, due to the usage of the far UV radiation (255 nm) for the fluorescence excitation, such method of tissue identification requires safety tests before it could be applied to visualize biological tissues *in vivo*.

The broad autofluorescence spectral region of the heart tissues extending to about 500 nm (Fig. 2) implied that not only the UVC radiation, but also the radiation at longer wavelengths could be applied to excite the fluorescence of endogenous fluorophores other than the residues of aromatic amino acids. Therefore, we aimed at the determination of such excitation wavelengths, which could be suitable for the discrimination of the particular heart tissues.

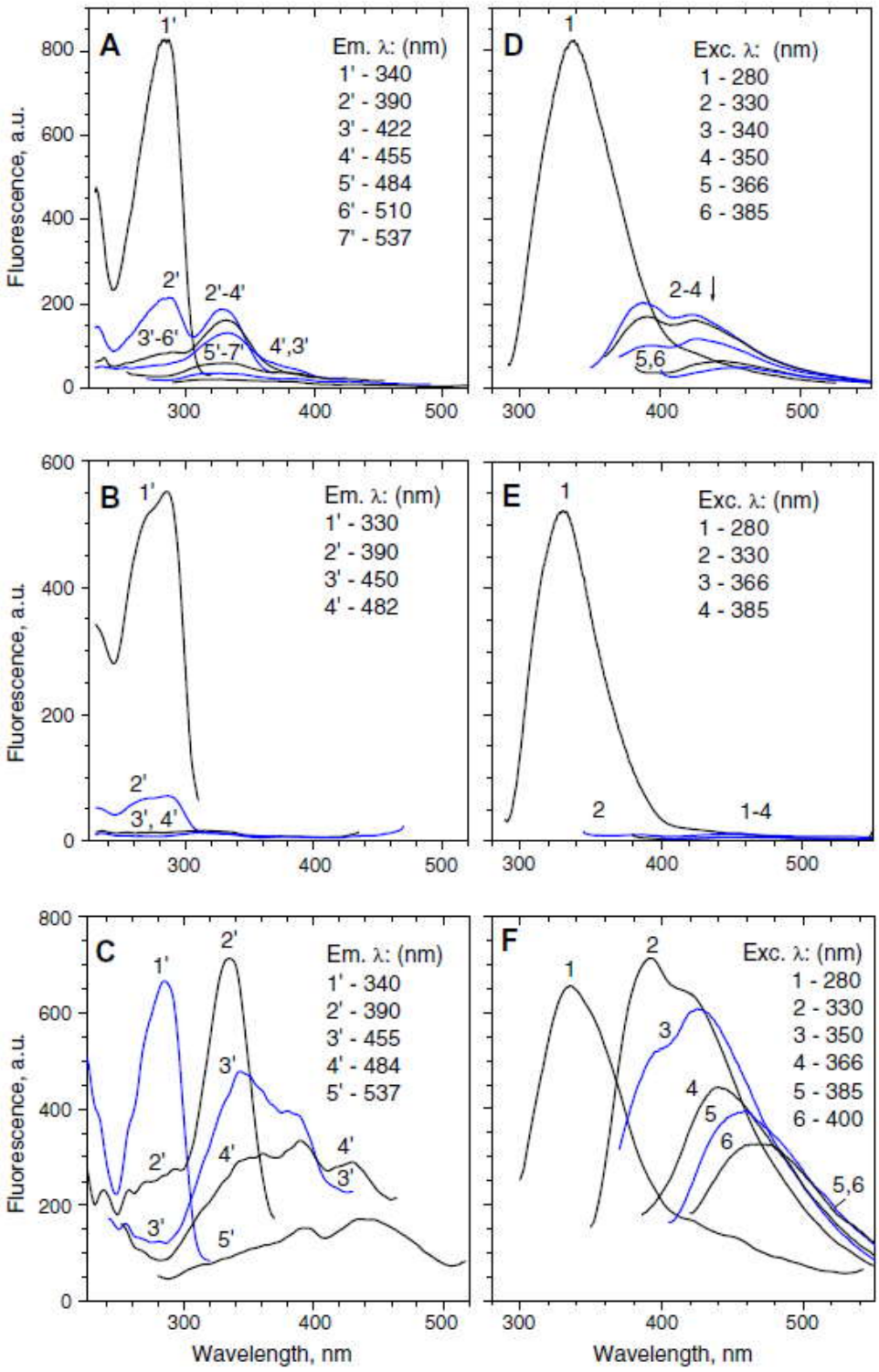

Fig.3. Fluorescence excitation (left column) and fluorescence emission (right column) spectra of the His bundle (top row: (A) and (D)), myocardium (middle row :(B) and (E)) and connective tissue (bottom row: (C) and (F)) specimens. Wavelengths chosen for the fluorescence excitation (Exc.) and the registration of the emission (Em.) are presented in the legends.

For this, the fluorescence spectra of the HB and myocardium (MC) tissues were registered by changing the excitation wavelength in small intervals starting from 280 nm to 385 nm (Fig. 3 D and E) and inspected for the differences in the spectral distribution of the fluorescence intensity. Since the human conduction system is embedded in the connective tissue, the analogous spectra registered from the specimens of connective tissue (CT) taken from the tricuspid heart valve were included for the comparative purposes (Fig. 3 F).

Initially, the broad band with a peak at about 390 nm with a valley at 410 nm was detected in the fluorescence spectra of HB (Fig. 3 D, curves 2 and 3). This valley seems to be caused by the blood absorption in this spectral region. Excitation at wavelengths longer than 340 nm resulted in redistribution of the intensity of this band (Fig. 3 D, curve 4) as well as a shift of the maximal fluorescence intensity towards longer wavelengths up to 460 nm (Fig. 3 D, curves 5 and 6). It should be noted that under the excitation in the spectral region, where aromatic amino acids do not absorb ($\lambda_{ex} > 330$ nm), the fluorescence intensity of HB remained significantly higher in the visible spectral region compared with that of the MC. One of the possible reasons for this might be a higher amount of some endogenous fluorophores, which are present in HB, while almost absent in the MC.

On the other hand, the fluorescence of CT was found to have the highest relative intensity in the visible spectral region (Fig. 3 F, curve 2). The shapes of the fluorescence spectra of HB and CT were quite similar under excitation at 330 nm; for instance, the ratios of the fluorescence intensities estimated at 390 nm and 460 nm differed less than 3%. The distinguishing features of CT fluorescence spectra (Fig. 3 F, curves 4 and 5) in comparison with the spectra of the HB (Fig. 3 D, curves 5 and 6) were the significantly higher relative intensity as well as much less pronounced decrease of its long-waved side under excitation at wavelengths longer than 330 nm.

To reveal the spectral properties of the fluorophores, which can cause the observed spectral differences, the fluorescence excitation spectra of the same samples have been registered. Fluorescence excitation spectra of HB (Fig. 3 A) and CT (Fig. 3 C) differed from those of the MC (Fig. 3 B). The excitation spectra

of the MC for both wavelengths of registration set at 330 nm and 390 nm (Fig. 3 B, curves 1' and 2') showed one distinct peak located at about 280 nm, which is compatible with the absorption band of Trp. The intensity of this band was high for all types of heart tissues when monitored in the spectral range typical for Trp fluorescence (330-340 nm). The excitation spectra of HB and CT registered at 390 nm (Fig. 3 A and C, curve 2') distinguished themselves by the appearance of a new band with a peak at 330 nm, which was more pronounced in the case of CT. The intensities of this band in the spectra of MC (Fig. 3 B) were much lower. Registration at longer wavelengths in the case of HB (Fig. 3 A, curves 3'-5') resulted in a reduced intensity as well as a slight shift and broadening of this band. The spectra of CT as compared with those of HB featured similar reduction in relative intensity at 330 nm and a shift of the maximum (Fig. 3 C, curves 3'-5') as well as the presence of another broad band with a maximal intensity at about 390 nm. The spectral features observed registering at longer wavelengths also included further bathochromic shift of the peak intensity. Similarly, as in the case of the emission spectra of CT, a sloping decay in intensity was detected at the long-waved side of the fluorescence excitation spectra, which extended up to 500 nm (Fig. 3 C, curve 5').

These results show that fluorescence excitation spectra of HB as well as CT in the range from 300 nm to 400 nm consisted of several overlapping bands, the intensities and peak positions of which varied depending on the wavelength chosen for the registration of fluorescence.

Thus, the fluorescence measurements performed on the heart tissues *ex vivo* imply that autofluorescence in the 390-550 nm spectral range is caused by several fluorophores with overlapping fluorescence bands. Their relative amounts in HB seem to be significantly higher than in MC but lower than in CT.

The band at 385 nm, which has been detected in the fluorescence excitation spectra of CT under registration of fluorescence at 455 nm orlonger wavelengths, could be attributed to elastin. It has not been observed in the spectra of other heart tissues. The analogous spectra of HB featured only the changes in the band at 330 nm, which is typical for the absorbance of collagen type molecules. These

differences in spectral features could be exploited for distinguishing between the specimens of HB and CT in the following way. The fluorescence intensities of CT measured at 460 nm or longer wavelengths had almost the same values under the excitation at 330 nm, 366 nm or 385 nm (Fig. 3 F, curves 2, 4 and 5) giving value for the ratio of the intensities close to one. In contrast, the corresponding values for fluorescence intensities of HB obtained under the same excitation conditions differed more than twice (Fig. 3 D, curves 2, 5 and 6).

Comparison of the fluorescence emission and excitation spectra with reference data [92] allow us to point out the endogenous fluorophores present in the heart tissues, which can influence their fluorescence properties in the visible spectral region.

The dominant endogenous fluorophores in CT are structural proteins: collagen and elastin. Five types of collagen (I, III, IV, V and VI) were found in heart tissues; type II collagen was not. Fibrous collagens of types I and III qualify as the most widespread structural proteins and comprise about 90 percents of dry weight of collagen in the myocardium.

The spectral properties of I, III and V type collagens are very similar (the databases of excitation-emission matrices, for Ref. see [92]). They can be excited in the 315-375 nm spectral range and show a broad emission band in the 360-440 nm range with the maximum at about 390 nm. Collagen of type IV absorbs in a far UV region (270-310 nm) and fluoresces at 315-375 nm with the maximum at 340 nm. The spectra of type VI collagen are shifted to the long-waved side (an absorption maximum at 410 nm and emission at 600-660 nm).

Elastin possesses a broad fluorescence band in the 370-560 nm range with a maximum at 440-450 nm under excitation in the range of 325-490 nm with the highest fluorescence intensity being excited at 360 nm. Fluorescence of NADH is less expected in specimens *ex vivo*.

The analysis of the fluorescence excitation and emission spectra resolved three dominant endogenous fluorophores with spectral peaks at 280/340 nm (excitation/emission), 330/390 nm and 385/ 460 nm: Trp, collagen and elastin, respectively. The observed differences of fluorescence intensity in the blue and UV regions typical for the MC, HB and CT spectra suggest a different relative content of the above mentioned fluorophores.

In summary, the comparison between the averaged fluorescence excitation spectra of HB and those of the MC tissue (Fig. 4 A) revealed that the maximal difference in intensity was typically observed under excitation in the spectral range from 320 to 370 nm, when spectra were recorded at 450 nm. As expected, the biggest difference in intensity between the fluorescence spectra of HB and the MC tissue was found between 420 and 465 nm under excitation at around 360 nm (Fig. 4 B). The fluorescence of CT can be distinguished from that of HB by comparison between the ratios of the fluorescence intensity excited at about 330 nm (absorption of collagen prevails) to that excited at 366 nm or 385 nm (where absorption of elastin prevails) measuring at 460 nm or longer wavelengths. The value of this ratio was expected to be about twice higher for HB than for CT.

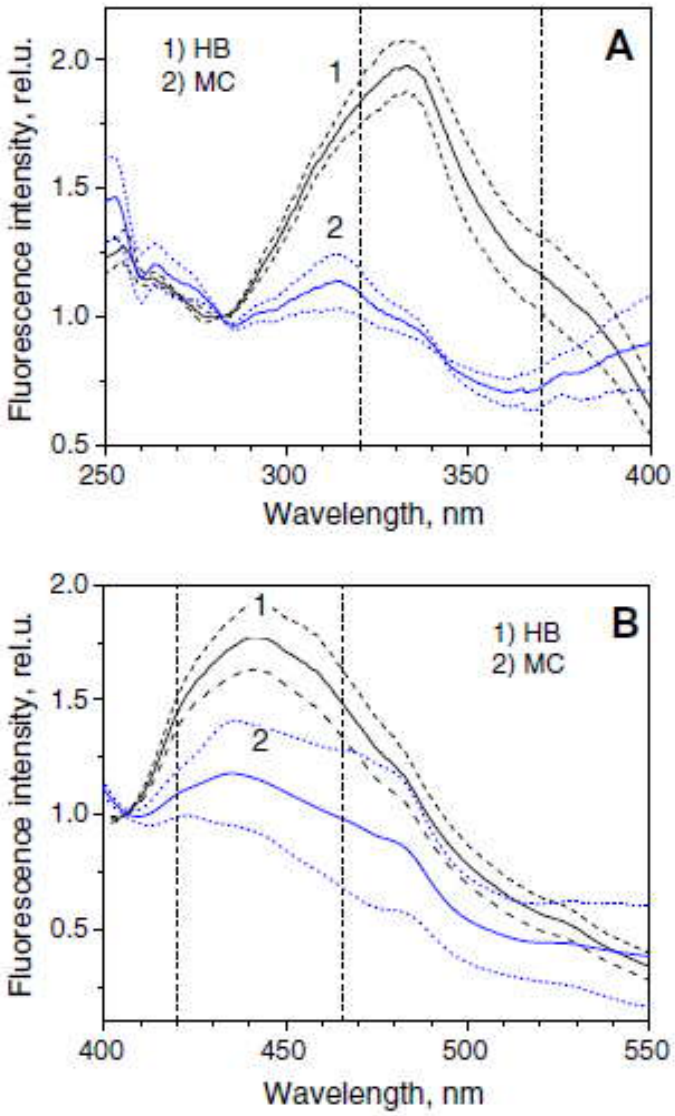

Fig. 4. Averaged fluorescence excitation spectra (λ_{em}= 450 nm, (A)) and fluorescence emission spectra (λ_{exc}= 360 nm, (B)) of His bundle and the myocardium normalized at 282 nm and 406 nm, respectively. Dotted lines mark the standard deviation.

Visualization experiments

Based on the different spectroscopic properties of the heart tissues as reported above the attempt to determine the presence and location of HB branches in the selected specimens was made by means of the fluorescence microscope and a digital camera. The places on the specimens yielding a more intense fluorescence, observed during the microscopic visualization, demonstrated good correlation with areas

attributed to HB branches during the morphologic analysis. Fig. 5 A depicts a typical specimen of the fragment of the left branch of HB with the surrounding endocardium, which was subsequently chosen for the spectral surface scanning measurements. The black line on the picture shows the direction and the length of the scanning path. Fig. 5 B illustrates the spectral fluorescence data obtained from the specimen under the same excitation and registration conditions. The peak fluorescence intensity of HB branch was more than twice higher than that of the surrounding endocardium.

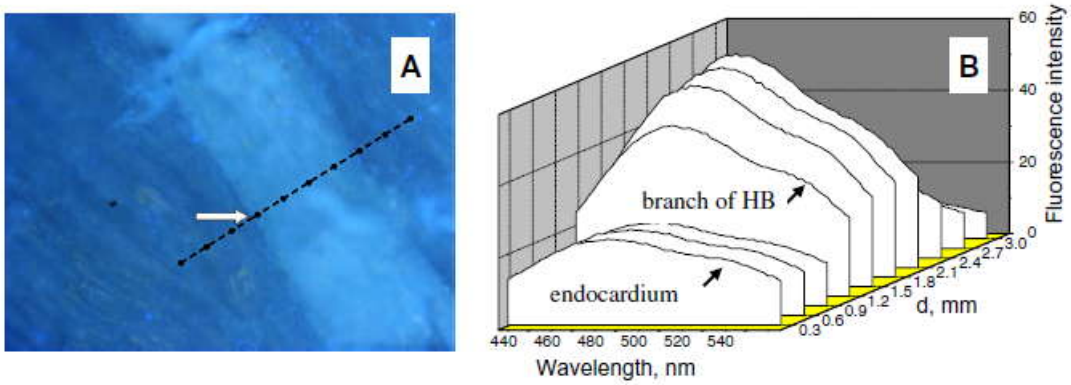

Fig. 5. The image (A) and fluorescence emission spectra (B) of the left His bundle branch fragment under illumination at around 366 nm. The fluorescence image and the fluorescence spectra have been obtained as described in the *Materials and methods* part using optical set-ups with the same spectral characteristics.

Preliminary experiments of the macroscopic visualization keeping the same excitation wavelength were initiated on the specimens of the whole interventricular septum. The image of such specimen taken by a CCD camera under usual daylight illumination presented in Fig. 6 A.

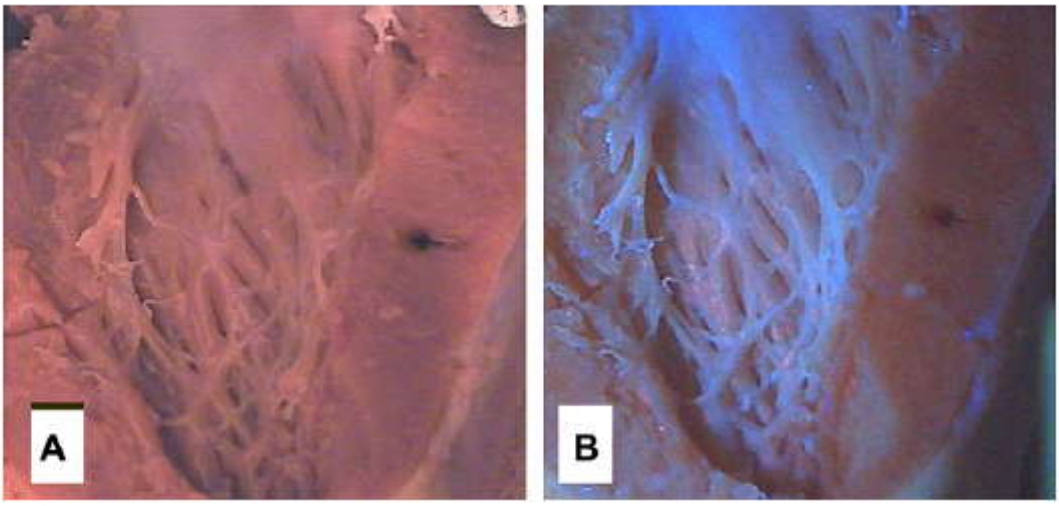

Fig. 6. The images of the left branch of HB in the interventricular septum, which had been exposed to daylight (A) and under 366 nm excitation (B), taken in a whole range of the visible spectrum without spectral filtering. UV illumination helps to visualize the presence of HB branch in the heart specimen by its blue fluorescence glow. The images taken with a digital CCD camera ToUcam Pro (Philips) and processed on a personal computer. The scale mark is 10 mm.

The branch of His bundle cannot be discerned under these conditions. Another image of the specimen (Fig. 6 B) was taken under illumination at around 366 nm. Here, the blue fluorescence of a furcated left branch of HB, which divaricates to the complex network of HCS, is visible at the top of the interventricular septum. The visualization of the specimens prepared from some other hearts, however, was not so distinct and demonstrated that the fluorescing areas observed under illumination at 366 nm were not limited to the autofluorescence of HB branch, but also included a relatively strong autofluorescence signal from the connective tissue present in the surrounding endocardium. The obtained spectroscopic data imply that in such cases the estimated variation in the fluorescence intensity under excitation at two selected wavelengths, at which absorbance of collagen prevails that of elastin and vice versa, could be of use for discrimination of His bundle branch.

The autofluorescence of His bundle, the myocardium and the connective tissue taken from tricuspid valve of the human heart was studied by using various excitation wavelengths. As it was found from both fluorescence excitation and

emission spectra the dominant fluorophores present in different amounts in these human heart tissues were tryptophan, collagen and elastin.

The fluorescence spectra recorded from the His bundle tissue under excitation at UVC region could be distinguished from those of the myocardium by more than two-fold increase in intensity at wavelengths from 400 to 500 nm. The clear difference between fluorescence signals also remains in this region under excitation at longer wavelengths (e.g., λ_{ex} = 330 nm, 350 nm or even 385 nm).

The comparison of the autofluorescence data of the connective tissue and the bundle of His, on the other hand, reveals the possibility of differentiation between these tissues based on the ratio of intensities measured at 460 nm or longer wavelengths under the predominant excitation of collagen at 330 nm and elastin - at longer wavelengths (e.g. 366 nm or 385 nm).

Investigation of the heart tissue extracts

In this study, the MC, HB and CT tissue extracts were fractionated by SDS-PAGE and the gels were investigated by spectroscopic methods with the aim of determining the localization of endogenous fluorophores in the fractions of these extracts. It was of interest, because the HB protein composition was investigated for the first time.

Heart tissues were disrupted and homogenized at several stages. The choice of composition for extraction buffers (see *Materials and methods*) was determined by the requirements of extensive solubility procedure and the extracts' compatibility for further spectro-photometric studies. Although treatment with nonionic detergents proved to be a very useful tool for the solubility of proteins, extracts containing Triton X-100 and/or Nonidet-40 showed a strong UV absorbance thus making spectro-photometric measurements complicated. However, even after treatment with high ionic strength buffers and with high levels of dissociating agents such as urea and SDS, some differences in fluorescence spectra of the remaining pellets from different tissues were observed (results not shown). Fig. 7 represents spectra of the first soluble fraction of heart tissues (fluorescence and fluorescence excitation spectra of all soluble fractions

were quite similar).

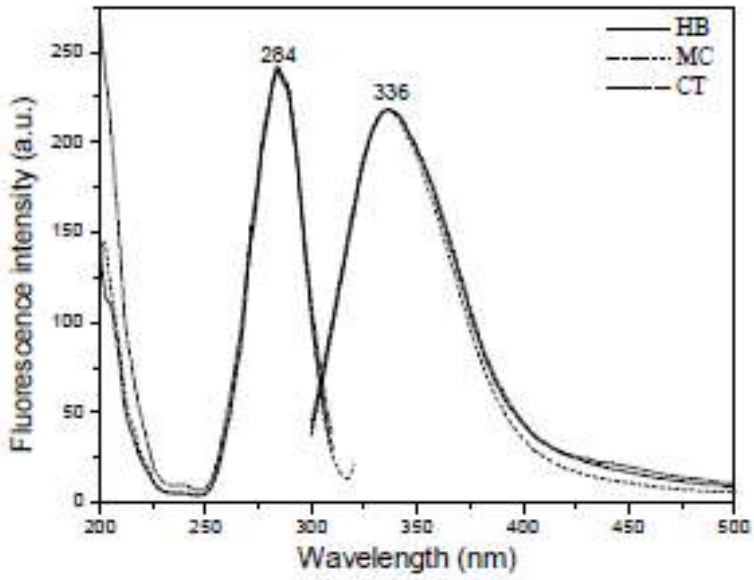

Fig.7. Fluorescence excitation spectra (λ_{fl} = 335) nm and fluorescence spectra (λ_{ex} = 280 nm) of His bundle, the myocardium and connective tissue homogenates (first soluble fraction) normalized at 284 nm and 336 nm, respectively.

To compare the spectra in their form, the obtained spectra were normalized. Normalization of two spectra was performed by multiplying the less intense one by a certain number that maximums of both spectra have the same intensity value. A characteristic band around 284 nm in the excitation spectra and a wide band around 336 nm in the fluorescence spectra were detected. This pair of excitation and emission bands could be assigned to tryptophan residues. Consequently, fluorescence of the soluble fractions is excitable only in the region of the absorption of aromatic amino acids. By exciting in the UVA range no fluorescence in the blue region was observed as in tissue samples from the myocardium and heart conduction system [85]. Thus, fluorophores responsible for emission in this spectral region precipitated out as an insoluble fraction proved by spectroscopic data. SDS-PAGE electrophoregrams of soluble fractions of heart tissues were composed of multiple bands of proteins (Fig. 8).

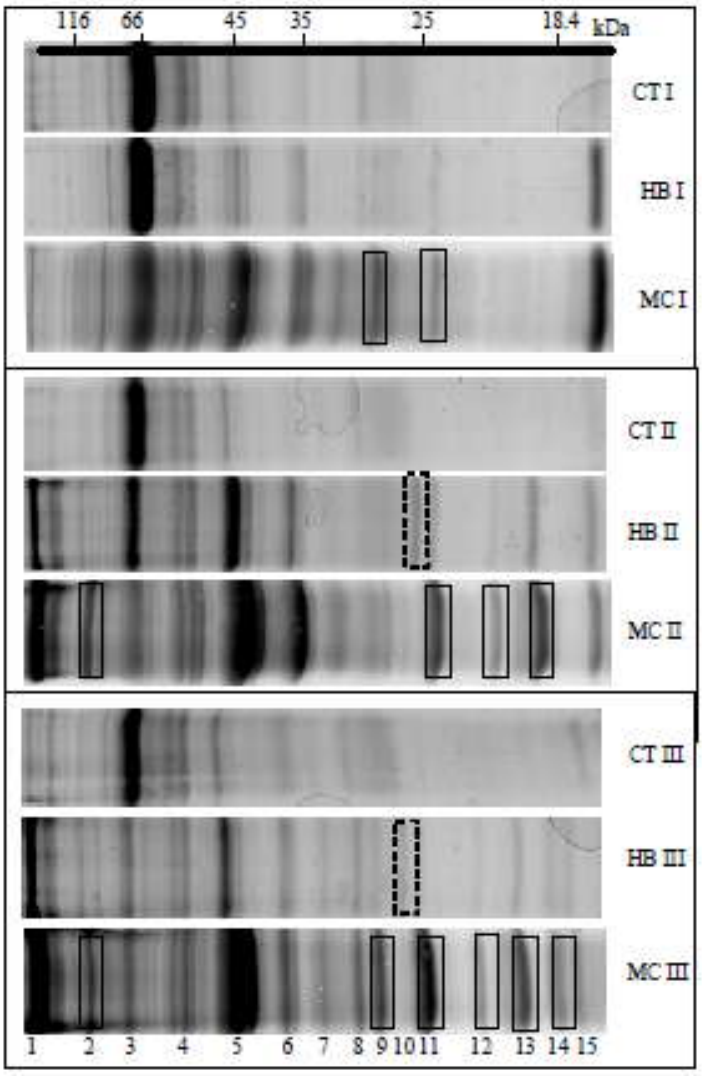

Fig. 8. SDS-PAGE of the heart tissue extracts. On the top – mass scale, at the bottom – numbers of protein groups. Numbers I, II. III represent the first, the second and the third fractions respectively. Groups of proteins marked by a solid line are specific to the myocardium, and groups specific to His bundle are marked by a dashed line.

Though electrophoresis results slightly varied depending on the tissue specimen and on how fine it was mashed, some regularity was observed. We distinguished 15 main protein groups according to differences in quantity between particular heart tissues. During the subsequent studies proteins of the same weight were characterized according to fluorescence and fluorescence excitation spectra. To this end, the four most apparent groups, noted as A, B, C and D, were chosen

(summarized in Table 2.).

Table 2. Proteins present in soluble fraction of the heart tissues studied by spectroscopic methods.

Group of proteins (No.)	Apparent molecular weight, kDa	Soluble fractions of CT			Soluble fractions of HB			Soluble fractions of MC		
		I	II	III	I	II	III	I	II	III
A (1)	~160–155					++	++		++	+++
B (3)	~66	+++	+++	+++	+++	++	+	++	+	+
C (4)	~57–52	+	+	+	+	+	+	++	++	++
D (5)	~45	+	+	+	+	++	++	++	+++	+++

The proteins (~160–155 kDa in weight) assigned to group A appeared only in soluble fractions II and III of HB and the MC. CT extracts contained negligible quantities of these proteins in soluble fractions. Therefore, these proteins seem to be specific for muscular tissues (MC and HB) and could be considered for differentiation between muscular and connective tissues. This group of proteins had very similar fluorescence and fluorescence excitation spectra as the protein group D (around 45 kDa). As is seen from Fig. 9 (160–155 kDa proteins (A) and 45 kDa proteins (D)), the excitation spectra had a typical band around 280 nm and a wide band around 328 nm in the fluorescence spectra.

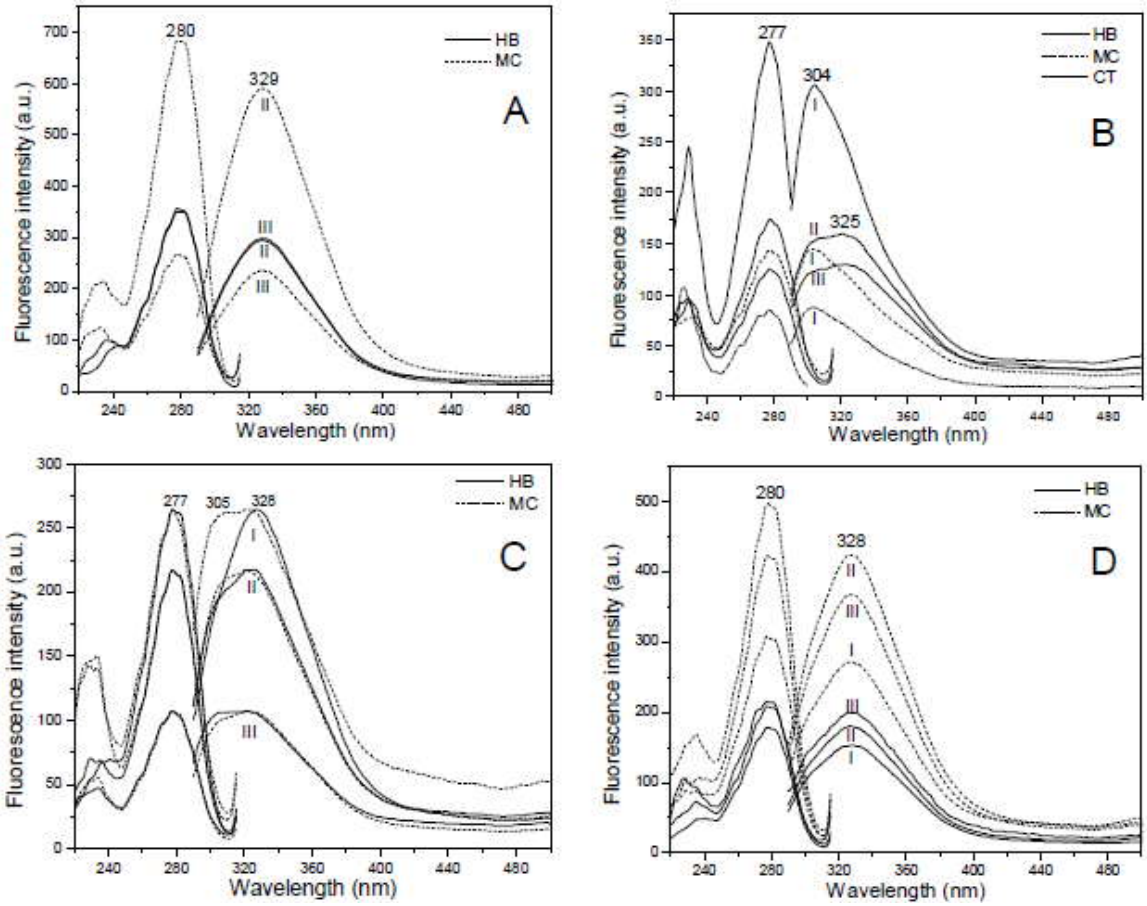

Fig. 9. Fluorescence excitation spectra (λ_{fl} = 329 nm) and fluorescence spectra (λ_{ex} = 270nm) of His bundle, the myocardium and connective tissue soluble fractions (I, II, III); groups of proteins: (A, 160-155 kDa) (A), (B, 66 kDa) (B), (C, 57-52 kDa) (C) and (D, 45 kDa) (D).

Again, this pair of bands can be attributed to W residue, the dominant fluorophore in proteins. The emission maximum of W in neutral solvent is around 310 nm, in water it occurs near 350 nm and is highly dependent upon polarity and the surrounding environment because of the presence of two overlapping electronic states [80]. Fluorescence bands observed around 328 nm indicated that in this case W residues had been localized inside the protein and shielded from solvent by the protein matrix. 270 nm was chosen for fluorescence excitation, because at this wavelength both fluorescent amino acids – tyrosine and tryptophan – are excited.

The protein group B (~66 kDa in weight) dominated in all fractions of CT and

in fraction I of HB. In fractions II and III of HB it appeared in smaller quantities. In the MC this strip appeared in all three fractions but was even less intensive than in HB. Interestingly, ~66 kDa proteins present in HB and CT soluble fractions were established as being strongly glycosylated while the MC peptides were not (results not shown). Thus, it may be assumed that proteins of the heart tissues having very close molecular weights are not identical. The spectra of this protein group were measured from fraction I of all tissues and from fractions II and III of HB (Fig. 9B). The results showed that fluorescence and fluorescence excitation spectra of fraction I were very similar for all tissues and had a fluorescence band at 304 nm, which can be assigned to tyrosine residue emission. Generally, the emission of proteins is determined mainly by tryptophan, which absorbs at the longest wavelengths and possesses the largest extinction coefficient. Also, due to such tryptophan absorption, the energy absorbed by tyrosine residues is often transferred to tryptophane residues in the same protein and the fluorescence of Tyr residues is strongly quenched. The fluorescence of Tyr residues could be observed only in proteins where Trp is absent or is located far from Tyr and there is no energy transfer (Forster's radius for the Trp–Tyr pair is 9 – 18 Å) [80]. The obtained results indicated that Trp is almost absent in fraction I proteins. A broad fluorescence band consisting of two overlapping bands around 304 nm and 325 nm was observed in the fluorescence spectra of fractions II and III of HB (Fig. 9B). Fluorescence around these wavelengths could be attributed to Tyr and Trp residues. Therefore, these proteins are different from those observed in fraction I. These protein bands in fractions II and III are composed of proteins containing both amino acids – Tyr and Trp.

The fluorescence spectra of 57–52 kDa proteins (group C, Fig. 9C) were obtained from fractions I, II and III of HB and the MC tissues. The spectra of each fraction were normalized for clarity. The main spectral differences were observed between samples of fraction I. A fluorescence band with a maximum around 328 nm was detected in HB proteins, indicating the presence of Trp in these proteins. The fluorescence spectra of proteins in the MC were composed of two overlapping bands with the maxima around 305 nm and 325 nm, implying that this protein group contains additional proteins in which W is absent or is far from

Tyr. Fluorescence spectra of fractions II and III of both HB and the MC were composed of two overlapping bands – around 305 nm and 325 nm, implying a similar composition of the proteins.

Several protein groups (2, 9, 11, 12, 13 and 14) were detected exceptionally in the second and in the third fraction of the MC as seen from SDS-PAGE data (Fig. 8). These proteins seem to be specific to the MC which could be distinguished from other tissues by staining one of these proteins. It is of interest to note that the protein group (10, ~26 kDa in weight) appeared only in fractions II and III of HB. In the present, we have no additional information on the identity of these peptides. However, this might be a protein or a group of proteins, which is specific only to HB. Labelling this protein with a specific dye or a fluorescing agent, the conduction system of the heart could be marked and easily visualized under appropriate illumination conditions.

2D gel electrophoresis

Proteomic analysis of a heart tissue is complicated by the large dynamic range of its proteins. In our studies, we have isolated total proteins from two human heart parts – the myocardium and the heart conduction system; proteins were fractionated by two-dimensional electrophoresis – one of the main technologies of proteomics. It is important to mention that this technology allows analysing a large spectrum of proteins and in our study it was performed in the pH range 3–10 IEF strips and SDS / PAGE gradient.

As one can see in Fig. 10, up to 200 polypeptides were detected by Coomassie staining in the human myocardium tissue (Fig. 10A). The number of total polypeptides in the human myocardium (Fig. 10A) was much higher than in the human heart conduction system (Fig. 10B).

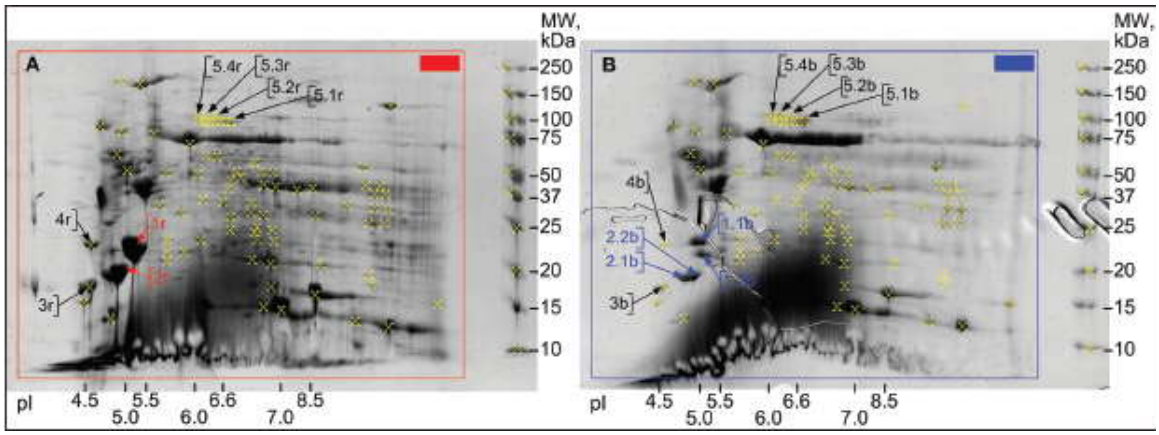

Fig. 10. Two-dimensional electrophoretic maps of proteins isolated from the human myocardium and heart conduction system. Total proteins isolated from heart tissues – the myocardium (A) and heart conduction system (B) were fractionated by 2-D electrophoresis, and the gels were stained by Coomassie Brilliant Blue G-250 (Fermentas, Lithuania). Molecular weight marker was from Fermentas, Lithuania. Crosses denote selected landmarks used for image registration. The positions of the spots selected for a detailed analysis are marked by arrows and numbers in the images. Spot labels ending with *r* were used only for Gel1 and in *b* for Gel2. Rectangles mark the area used for automatic spot detection. MW markers have been kept (on the right side of images).

We have found that proteins nos. 1, 2, 3 and 4 (Figs. 10-11) were much more pronounced in the human myocardium tissue than in the human heart conduction system. Only the expression of protein no. 5 was lower in the heart conduction system.

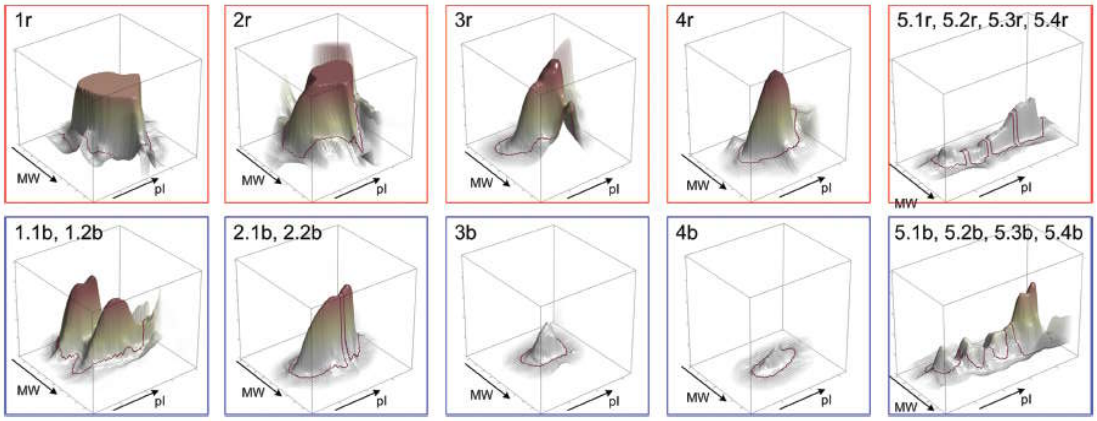

Fig. 11. 3-D plots of the selected spots. The 3rd dimension is the intensity of gel image area around a spot. 1r, 2r, 3r, 4r, 5.1r, 5.2r, 5.3r ir 5.4r – MC proteins groups. 1.1b, 1.2b, 2.1b, 2.2b, 3b, 4b, 5.1b, 5.2b, 5.3b ir 5.4b – HB proteins groups. MW – molecular mass, pI – isoelectric point.

Changes in protein expression level in the human myocardium and heart conduction system reveal the necessity to identify proteins especially specific for the heart conduction system.

Discussion

Conduction system of the human heart as a unanimous anatomical structure was described in the beginning of the 20th century. L.Aschoff and J.G.Monckeberg defined main morphologic criteria for identifying this system. For the investigation of the human heart conduction system macroscopical and histological methods were used. A macroscopical investigation method is simpler, it is less time and money consuming; therefore, many researchers used this method (M. Holl 1911 m., E.W. Walls 1945 m., M. Lev 1951 m.) and various modifications (Sitnikov V. A. 1972 m., Umovist M.I. 1973 m. Siniov A.F., Krimski L.D. 1985m.). Unfortunately, macroscopical investigations not always were successful. K. Reemtsma in 1958 set up preparation of 11 healthy hearts and only in three hearts the atrioventricular node and His bundle were found. M.J. Davies in 1971 after unsuccessful attempts enounced that it was impossible to

find the conduction system of the heart by macroscopical method and started to use histological method only, like the majority of other investigators (S.Bharati 1978, J. Windran 1951; R.C.Truex 1958.). When this method was rejected by the majority of authorities the macroscopical preparation of the conduction system was neglected. Nowadays descriptions of the human heart conduction system are based on investigations of the histological slides and anatomical diagrams created according to histological investigation data. It is impossible to prove precision of these diagrams because anatomical heart preparations were destroyed at histological investigation. Tissue blocks from the heart regions where usually is stated the conduction system, or where a researcher expects to find this system, are usable for histological investigation. If these blocks do not contain the conduction system, a researcher must investigate the whole heart by serial histological sections, prepare and examine thousands of histological slides. The macroscopical preparation method demands preparation skills but gives some advantage. During the macroscopical preparation it is possible to evaluate the condition and location of the whole ventricular conduction system. In the case of pathology it enables to take tissue samples for histological investigation. In such situation few histological slides are satisfactory to evaluate changes and there is no necessity to prepare thousands of slides. For these reasons, more profitable is usage of the macroscopical investigation method with histological evaluation of small tissue blocks. The macroscopical preparation method may be modified according to investigation purposes. For example, if surgical injury of His bundle is suspected, it is not necessary to investigate the sinus or atrioventricular nodes, distal parts of His bundle. Such method facilitates investigation of the conduction system but is usable only in correctly developed hearts. In the hearts with congenital anomalies the macropreparation method is hardly utilizable. Without available anatomical landmarks macropreparations of the conduction system are almost impossible. The macropreparation method allows getting a pure conduction system tissue, not processed chemically. Undoubtedly, conduction system tissue samples may be extracted from paraffin blocks, but such samples have undergone fixation, paraffin embedding and deparafinisation procedures. Tissue processing procedures may influence results of future investigations.

Therefore, the investigation of pure and chemically unaffected conduction system tissues was performed for the first time. Originally, there were compared investigation results of formalin fixed and unfixed tissue samples in pursuance of finding stable morphological differences.

Distribution and concentration of potassium, calcium, sodium and chlorine ions is very important for bioelectrical impulse generation, therefore, X-ray electron microscopy (SEM/EDX method) investigations of the heart tissues were performed. This method allows estimating essential composition of the exploratory material. His bundle and ventricular myocardium tissue samples show the same amount of phosphorus, chlorine, calcium, sulphur, silicon, sodium and potassium ions. Thus, velocity of the bioelectrical impulse spreading in His bundle is not related to ions concentration differences.

Differently prepared tissue samples were compared by FT-IR absorption spectroscopy method. Differences between formalin fixed and dried tissue samples were not established. Investigation of the formalin unfixed and nondried His bundle and ventricular myocardium tissue samples show the same differences. Formalin fixation and desiccation have no influence on stabile, structural proteins; therefore, differences between differently prepared tissues possibly are determinate by changes of free cytoplasmic molecules. Such assumptions are indirectly confirmed by the finding, that the ventricular myocardium may contain more free or minus charged (α-asparagines and α-glutamic) amino acids. FT-IR absorption spectra of biological tissues are the sum of protein amino acids and free amino acids spectra, therefore, specific bands of His bundle and the myocardium FT-IR spectra cannot be labeled to particular molecule groups and it is impossible to determine reasons of the differences. This does not admit that the differences are entirely related to free amino acids. Differences between His bundle and the myocardium FT-IR spectra allow definitely separating these tissues, but are hardly usable in practice for complexity of this method.

UV absorption spectroscopy also confirms differences between the His bundle and myocardium tissues. Different amounts of aromatic amino acids were demonstrated. His bundle contains more Trp 4 times and Tyr 2, 5 times than

myocardium. Formalin fixed and paraffin embedded tissue samples for UV absorption spectroscopy were used, therefore, it may be believed that aromatic amino acids, which determine these differences, are embedded in stable, structural proteins. Different amounts of aromatic amino acids in the His bundle and myocardium may be related to a different function of these tissues. Spectral differences were noted at the short UV-B (280) bands region, which is dangerous for biological tissues and may cause burns. For this reason, in practice UV absorption spectroscopy is useless.

Histochemical methods do not show differences of Trp and Tyr amounts in His bundle and myocardium tissues. As show UV absorption spectra, molecules of aromatic amino acids are in more polar environment, available for water in pancreas. Trp molecules in His bundle are in less polar environment, which determine displacement of the spectrum towards a short spectrum bands side. It means that aromatic amino acid molecules are less available for water and may be the reason of negative results of histochemical staining.

Differences between human His bundle and the myocardium were also demonstrated by specific fluorescence and excitation spectra. According to fluorescence intensity near 340 nm and 450 nm regions with excitation in aromatic amino acid absorption region (250-290 nm) are possible unambiguously to separate His bundle and myocardium tissues. Unfortunately beams of the excitation region are dangerous for biological tissues, therefore, this method would be difficult to use in practice for *in vivo* or routine investigations. Various endogenous fluorophores of the human heart (collagen, elastin, NADH, flavins, porphyrins, lipopigments and glycosaminoglycans) may fluoresce at the blue spectrum region excited not only by a short UV region (280-100 nm.) but by a longer beam too. Each fluorophore distinguished by excitation and emission spectra, and registration of the fluorophore spectra in wide spectral region allow identification of each of fluorophores. In this investigation, we used a wide excitation region from 250 nm to 480 nm. Such broad excitation region allowed finding of more effective excitation regions for visualization experiments [94].

Analysis of the heart tissue fluorescence and excitation spectra reveals some endogenous fluorophores with similar absorption and fluorescence bands. Three

leading endogenous fluorophores were estimated: tryptophan with excitation/fluorescence bands near 280/340 nm, collagen with excitation/fluorescence bands near 330/390 nm, and elastin 385/460 nm. Different amounts of these fluorophores in the myocardium, His bundle and the connective tissue determine distinctions of the fluorescence spectra of these tissues in UV and blue regions. Very slack blue fluorescence of the myocardium may be explained by a small amount of collagen and elastin in the myocardium in comparison with His bundle and the connective tissue. Exceptionally clear spectral bands of these fluorophores are seen in homogenate spectra of the heart tissue.

In cases when intensive blue fluorescence of the endocardium connective tissue aggravates the detachment of His bundle branches, fluorescence must be excited with two different lengths of the bands: 330 nm (collagen absorption region) and 385 nm (elastin absorption region). His bundle and the connective tissue differ by intensity of elastin fluorescence in 460 nm region. Intensity of the connective tissue fluorescence remains uniform using excitation at 330 nm and 385 nm, whereas fluorescence intensity of His bundle tissue decreases twice using excitation at 385 nm. According to these differences it is possible to create the human heart conduction system visualization model. Such model demands to register spectra excited by two different lengths of the bands at many points of endocardium and in diagram form delineate course of the conduction system. Such visualization model needs quite complex equipment and a lot of time.

Fluorescence of Trp and Tyr residues dominate in all soluble fractions of the heart tissue homogenates, whereas endogenous fluorophores with fluorescence in a visible spectral region remain in insoluble fractions. Although spectra of all soluble fractions of the heart tissues have no substantial differences, spectra of different protein groups, separated by electrophoresis method, are diverse. Even proteins with close mass distinguish by different fluorescence spectra, and this means that these soluble fraction proteins have got various structures.

Protein groups (~26 kDa), revealed by the electrophoresis method, are observed only in II and III fractions of His bundle. In fraction I, which contains water-soluble proteins, this group is not seen. Thus we may conclude that these

are insoluble, structural, membranous proteins, which differ from the known gap junction (40 kDa, 43 kDa and 45 kDa) and HCN (from 61 kDa to 120 kDa) proteins. Therefore, at this moment it is hardly possible to estimate dependence of these proteins.

First 2D gel electrophoresis investigations also show clear proteomic differences between the conduction system and myocardium tissues. Total amount of polypeptides was greater in the myocardium than in conduction system tissue. Discrete groups of the conduction system proteins are not homogeneous but binary, differently from the myocardium.

Performed investigations show different proteomic composition of the myocardium and conduction system. Which proteins are specific for the human conduction system, what physiological function they perform and where they are located – this remains not understandable. First visualization experiments show that it is possible to highlight the conduction system by optical methods on the background of other heart tissues. However, created visualization methods are rather complex at this moment and useless in practice. It is possible that future circumstantial proteomic investigations may permit identification and examination of proteins specific for the human heart conduction system. Specific bookmark of these proteins allows creation of the simple and usable visualization method of the human heart conduction system.

Conclusions

1. The arranged human heart conduction system preparation method allows to get a pure conduction system tissue and to provide the tissue for spectroscopical and proteomic investigations.

2. Spectroscopical investigations of the human heart conduction system and other heart tissues show:

a. X-ray electron microscopy investigation does not show any differences between myocardium and conduction system tissues. Examination results show that the tissues of the myocardium and conduction system contain equal amount of phosphorus, calcium, chlorine, sulphur, silicon, natrium and potassium ions.

b. Analysis of FT-IR absorption spectra indicates differences between myocardium and conduction system tissues. FT-IR method shows that the ventricular myocardium contains more free or minus charged (α-asparagines and α-glutamic) amino acids.

c. UV absorption spectra show clear differences between conduction system and myocardium tissues. These differences are determined by a various amount of aromatic amino acids.

d. Analysis of fluorescence and fluorescence excitation spectra displays three endogenous fluorophores (tryptophan, collagen and elastin) with similar absorption and fluorescence bands. Different amounts of these fluorophores determine spectral differences between conduction system, myocardium and connective tissue.

e. His bundle and the branches can be separated from the connective tissue by the fluorescent spectroscopy method. Fluorescence of these tissues must be excited by the light of two different lengths: 330 nm (collagen absorption) and 380 nm (elastin absorption) and following changes of fluorescence intensity near 460 nm. Fluorescence intensity of the connective tissue remains the same under both excitation beams, while fluorescence intensity of His bundle decline twice under excitation at 380 nm.

f. Analysis of the heart tissue homogenates fluorescence spectra shows that fluorescence in the blue region depends on insoluble structures.

3. Albuminous composition of His bundle and the myocardium differs. Electrophoresis shows protein groups (~26 kDa), which may be detected only in II and III fractions of His bundle.

4. Estimated fluorescence and proteomic differences between His bundle and myocardium tissues allow us to suggest that distinction of the bioelectrical impulse velocity in these tissues is determined by the specific morphological odds. According to these differences it is possible to create the visualization method of the conduction system.

Santrauka

Žmogaus širdies laidžioji sistema yra specializuotas raumeninės kilmės audinys, kuris generuoja ir perduoda bioelektrinį impulsą, sinchroniškai sužadinantį atskirų miokardo sričių susitraukimą. Žmogaus širdies laidžiosios sistemos komponentai randami visose širdies srityse, todėl dauguma patologinių procesų neišvengiamai pažeidžia šią sistemą, trikdydami sinchronišką miokardo sužadinimą ir efektyvų širdies darbą. Impulso plitimas gali sutrikti bet kurioje žmogaus širdies laidžiosios sistemos dalyje: sinusiniame mazge, atrioventrikuliniame mazge ir Hiso pluošte ar vienoje iš Hiso pluošto šakų. Vaikų širdies ritmo sutrikimai dažniausiai atsiranda dėl įgimtų laidžiosios sistemos anomalijų ir ektopinių šios sistemos židinių. Įgimti papildomi laidumo takai (Kento pluoštas) sukelia Wolff-Parkinson-White sindromą ir išsivysčius supraventrikulinėms tachikardijoms gali būti staigios mirties priežastimi. Širdies ligos ir jas komplikuojantys laidumo ir ritmo sutrikimai yra viena iš dažniausių mirties priežasčių civilizuotame pasaulyje. Apie 12% staigios kardialinės mirties atvejų anatominiai, mikroskopiniai ir biocheminiai širdies tyrimai nepaaiškina mirties priežasčių.

Kaip atskira struktūra žmogaus širdies laidžioji sistema aprašyta XX amžiaus pradžioje, tačiau dar penkis dešimtmečius vyko diskusijos ar ši sistema, tiksliau skilvelinė jos dalis, egzistuoja kaip atskiras anatominis žmogaus širdies darinys. Šias diskusijas lėmė tai, kad morfologiniai žmogaus širdies laidžiosios sistemos tyrimai yra ganėtinai sudėtingi. Tiek makroskopiškai, tiek histologiškai žmogaus širdies laidžiąją sistemą sunku atskirti nuo aplinkinių audinių, todėl siekiant įrodyti, kad tiriamojo audinio fragmentas priklauso ŠLS, būtina rasti jo ryšį su kitomis laidžiosios sistemos dalimis, t.y. būtina surasti ir ištirti visą skilvelinę širdies laidžiąją sistemą, o tai reikalauja įgūdžių, didelių laiko ir darbo sąnaudų. Dėl šių techninių tyrimo sunkumų, žinios apie žmogaus širdies laidžiąją sistemą yra negausios ir fragmentiškos, dažniausiai apsiriboja atskirų atvejų aprašymais, morfologiniai radiniai prastai koreliuoja su klinikiniu vaizdu. Šiuo metu vis dar nėra vieningos nuomonės ar egzistuoja anatominiai laidumo takai tarp sinusinio ir atrioventrikulinio širdies laidžiosios sistemos mazgų, žmogaus širdies laidžiosios sistemos ir atskirų jos dalių matmenys literatūroje pateikiami labai skirtingi. Aprašyti anatominiai variantai, įgimtos ŠLS anomalijos, tačiau jų dažnumas ir

pasiskirstymas nėra žinomi. Daugelio įgimtų širdies ydų atvejais, žmogaus širdies laidžiosios sistemos išsidėstymas išvis nėra žinomas. Žmogaus, mirusio dėl širdies laidumo sutrikimų širdyje dažnai nepavyksta rasti jokių svarbesnių patologinių širdies laidžiosios sistemos pakitimų, ir atvirkščiai – ryškūs patologiniai pokyčiai aptinkami širdyse tų žmonių, kurie niekada nesirgo širdies laidumo ir ritmo sutrikimais.

Tokius duomenų neatitikimus galima paaiškinti sudėtingomis žmogaus širdies laidžiosios sistemos tyrimo metodikomis. Anatominis šios struktūros preparavimas daugeliui tyrėjų pasirodė per daug sudėtingas ir šiuo metu beveik nebenaudojamas. Žmogaus širdies laidžiosios sistemos aprašymai ir tyrimai paremti iš histologinių pjūvių rekonstruotomis topografinėmis schemomis. Patikrinti, kiek šios schemos atitinka tikrovę neįmanoma, nes histologinių pjūvių gamybos metu sunaikinamas anatominis širdies preparatas.

Morfologiniai skirtumai, kurie leistų atskirti žmogaus širdies laidžiąją sistemą nuo miokardo, literatūroje aprašomi taip pat prieštaringai. Vienų tyrėjų aprašytų skirtumų kitiems dažnai nepavyksta patvirtinti, ir tokį duomenų nesutapimą galima paaiškinti skirtingų žmogaus širdies laidžiosios sistemos dalių analize. Pavyzdžiui, atrioventrikuliniame mazge gausu nervinių rezginių, turinčių sukcinatdehidrogenazės ir cholinesterazės, kitose širdies laidžiosios sistemos srityse nervinių rezginių su šiomis medžiagomis nėra.

Šiuo metu nėra sukurta tinkama žmogaus širdies laidžiosios sistemos vaizdinimo metodika, kuri palengvintų ne tik embriologinius, anatominius šios sistemos tyrimus, bet ir klinikinę praktiką. Operacijos metu, stovint širdžiai ir nesant elektrinės veiklos, elektrofiziologiniais metodais neįmanoma nustatyti širdies laidžiosios sistemos lokalizacijos, netipiškas laidžiosios sistemos išsidėstymas gali tapti jos chirurginio pažeidimo priežastimi su sunkiomis pooperacinėmis komplikacijomis. Žmogaus širdies laidžiosios sistemos vaizdinimas operacijos metu padėtų išvengti šios sistemos pažeidimų.

Nors žmogaus širdies laidžiosios sistemos anatomija ir histologinė struktūra aprašyta daugiau nei prieš 100 metų, šiuo metu tikslinga peržiūrėti, patikslinti ir papildyti šias žinias. Šiam tikslui pasiekti būtina turėti specifišką, patikimą ir nesudėtingą, tinkamą kasdieniniam naudojimui, žmogaus širdies laidžiosios sistemos vaizdinimo metodiką.

Tyrimo tikslas

Nustatyti morfologinius ir spektrinius skirtumus tarp žmogaus širdies laidžiosios sistemos, miokardo ir širdies jungiamojo audinio bei naudojantis šiais skirtumais sukurti žmogaus širdies laidžiosios sistemos vaizdinimo metodiką.

Tyrimo uždaviniai

1. Paruošti žmogaus širdies laidžiosios sistemos preparavimo metodiką, leidžiančią gauti širdies laidžiosios sistemos audinį be kitų širdies audinių ir pateikti žmogaus širdies audinių mėginius spektroskopiniams bei proteominiams tyrimams.
2. Ištirti žmogaus širdies laidžiosios sistemos ir kitų širdies audinių rentgenoelektroninius, infraraudonosios ir ultravioletinės (UV) sugerties bei savitosios fluorescencijos emisijos ir fluorescencijos žadinimo spektrus ir nustatyti parametrus ir spektrines sritis kuriose stebimi ženklūs žmogaus širdies laidžiosios sistemos ir kitų žmogaus širdies audinių skirtumai, naudotini širdies laidžiosios sistemos vaizdinimui širdies audinių fone.
3. Elektroforezės metodu palyginti žmogaus širdies laidžiosios sistemos ir kitų širdies audinių homogenatų baltyminę sudėtį bei jų spektrines savybes ir užregistruoti baltymų sąstato skirtumus.
4. Apibendrinus eksperimentinių tyrimų rezultatus nustatyti statistiškai patikimus skirtumus tarp žmogaus širdies laidžiosios sistemos ir kitų širdies audinių ir įvertinti jų panaudojimo galimybes kuriant laidžiosios sistemos vaizdinimo metodiką.
5. Atlikti žmogaus širdies laidžiosios sistemos vaizdinimo eksperimentus.

Ginamieji teiginiai

1. Širdies laidžiojoje sistemoje bioelektrinio potencialo atsiradimui svarbūs yra jonai, tačiau atlikti tyrimai rentgenoelektroniniu mikroskopu, parodė, kad fosforo, kalcio, chloro, sieros, silicio, natrio ir kalio kiekiai žmogaus širdies

laidžiosios sistemos Hiso pluošte (HP) ir miokarde (MK) paklaidų ribose yra vienodi. Todėl rentgenoelektroninės mikroskopijos metodas neleidžia atskirti žmogaus širdies laidžiąją sistemą nuo miokardo.

2. Skirtumai, nustatyti Hiso pluošto ir miokardo mėginių Furje transformacijų infraraudonosios (FT-IR) sugerties spektruose, leidžia daryti išvadą, kad žmogaus širdies laidžiosios sistemos Hiso pluoštas ir miokardas turi morfologinių skirtumų. Vienareikšmiškai žmogaus širdies laidžiosios sistemos ir miokardo mėginių FT-IR sugerties spektrų juostas priskirti konkrečių molekulių grupių virpesiams ir įvertinti skirtumų priežastis nėra įmanoma, nes biologinių audinių ir infraraudonosios sugerties spektrai yra amino rūgščių, priklausančių baltymams, peptidams ir laisvoms amino rūgštims, spektrų visuma, vienareikšmiškai nesusieta su audinio morfologija. Tačiau naudojantis IR spektro juostų ties 1450 cm^{-1} ir 1400 cm^{-1} pralaidumų santykiais galima tiksliai atskirti žmogaus širdies laidžiąją sistemą nuo miokardo.
3. Žmogaus širdies laidžiosios sistemos Hiso pluošto ir miokardo UV sugerties spektrai skiriasi savo intensyvumu aromatinių aminorūgščių sugerties srityje nuo 250 nm iki 300 nm. Nustatyta, kad žmogaus širdies Hiso pluošte triptofano yra apie 2, o tirozino apie 4 kartus daugiau negu miokarde. Vaizdinimui naudojant šioje spektro srityje spinduliuojančius šviesos šaltinius galima tikėtis skirtingos širdies audinių fluorescencijos, o tuo pačiu ir galimybės optiškai registruoti žmogaus širdies laidžiosios sistemos vaizdą kitų širdį sudarančių audinių fone.
4. Didžiausi žmogaus širdies laidžiosios sistemos Hiso pluošto ir miokardo savitosios fluorescencijos spektrų intensyvumų skirtumai stebimi 420-465 nm srityje, žadinant 320 – 370 nm srityje.
5. Žmogaus širdies laidžiosios sistemos Hiso pluoštą ir jo šakas galima spektroskopiškai atskirti nuo širdies jungiamojo audinio žadinant kolageno (330 nm) ir elastino (385 nm) sugerties juostose ir registruojant fluorescencijos intensyvumo pokyčius ties 460 nm.
6. Atlikus elektroforezę Hiso pluošto audinyje rasta apie 26 kDa molekulinės masės baltymų grupė, kurios nėra miokarde ir širdies jungiamąjame audinyje.

Rezultatų aptarimas

Širdies laidžioji sistema, kaip vieninga anatominė struktūra, aprašyta praėjusio šimtmečio pradžioje. Tada L.Aschoff ir J.G.Monckeberg nurodė pagrindinius morfologinius kriterijus, būtinus atpažinti širdies laidžiąją sistemą. Žmogaus širdies laidžioji sistema pradėta tirti makroskopiniu ir histologiniu metodais. Makroskopinis tyrimas yra paprastesnis, užima mažiau laiko ir kaštų, todėl daugelis širdies laidžiosios sistemos tyrinėtojų naudojo anatominį preparavimo metodą (M. Holl 1911 m., E.W. Walls 1945 m., M. Lev 1951 m.) ir įvairias modifikacijas (Sitnikov V. A. 1972 m., Umovist M.I. 1973 m. Siniov A.F., Krimski L.D. 1985m.). Deja, makroskopiniai tyrimai ne visada būdavo sėkmingi. K. Reemtsma 1958 m. preparuodamas 11 normaliai susiformavusios širdies preparatų tik 3 rado atrioventrikulinį mazgą ir Hiso pluoštą. M.J. Davies 1971 m. po nesėkmingo bandymo paskelbė, kad anatomiškai išpreparuoti širdies laidžiąją sistemą neįmanoma ir perėjo prie histologinio tyrimo, kaip ir daugelis kitų (S.Bharati 1978m., J. Windran 1951m.; R.C.Truex 1958m.). Daugumai autoritetų atsisakius anatominio širdies laidžiosios sistemos tyrimo, šis preparavimo metodas buvo nepelnytai užmirštas. Šiuo metu širdies laidžiosios sistemos aprašymai pagrįsti histologinių pjūvių tyrimu ir rekonstruotomis topografinėmis schemomis. Patikrinti šių schemų tikslumą neįmanoma, nes jas kuriant sunaikinamas anatominis širdies preparatas. Audinių blokai histologiniam tyrimui imami iš tų širdies sričių, kuriose yra tipiškai išsidėsčiusi širdies laidžioji sistema, arba kur tyrėjas tikisi ją rasti. Jei paimtuose blokuose laidžiosios sistemos rasti nepavyksta, belieka serijiniais pjūviais tirti visą širdį, t.y. paruošti tūkstančius histologinių preparatų ir juos ištirti. Makroskopiam laidžiosios sistemos preparavimui reikia įgūdžių, tačiau šis metodas turi nemažai privalumų. Makroskopinio preparavimo metu galima įvertinti visos skilvelinės ŠLS dalies būklę, išsidėstymą ir esant patologijos įtarimui, paimti audinio fragmentus histologiniam tyrimui, t.y. tokiam tyrimui užtenka paruošti tik kelis histologinius preparatus, tuo metu kai tiriant širdies laidžiąją sistemą histologiniu būdu, būtina visą tiriamą audinio bloką supjaustyti serijiniais pjūviais, t.y. paruošti kelis tūkstančius preparatų ir juos ištirti. Dėl šių priežasčių geriausiai

taikyti makroskopinio preparavimo metodą histologiškai tiriant tik tam tikras širdies laidžiosios sistemos sritis, taip sutaupant laiką ir išlaidas. Makroskopinio preparavimo metodiką galima modifikuoti, atsižvelgiant į tyrimo tikslus ir poreikius. Pavyzdžiui, įtariant operacinį Hiso pluošto pažeidimą galima netirti SA ar AV mazgų, distalinių Hiso pluošto šakų sričių. Tokia metodika labai palengvina ŠLS tyrimus, tačiau yra tinkama įvertinti tik normaliai suformuotos širdies laidžiosios sistemos būklę. Esant įgimtoms širdies ydoms ar širdies laidžiosios sistemos anomalijoms preparuoti ŠLS yra labai sudėtinga. Nesant tinkamų anatominių ŠLS išsidėstymo žymenų, kurie naudojami normaliai suformuotose širdyse su tipiškai išsidėsčiusia laidžiąja sistema, makroskopinis preparavimas beveik neįmanomas.

Makroskopinė preparavimo metodika taip pat leidžia gauti švarų, cheminėmis medžiagomis nepaveiktą ŠLS audinį. Be abejo, ŠLS audinį galima gauti iš parafininio bloko, tačiau toks mėginys jau bus fiksuotas, įlietas į parafiną, vėliau deparafinizuotas. Audinio apdorojimas gali turėti įtakos tolimesnių tyrimų rezultatams. Be to, makroskopinio preparavimo metu paprastai pavyksta be aplinkinių audinių fragmentų paimti Hiso pluoštą ir proksimalinę dešinės šakos dalį. Gautas švarus, be aplinkinių audinių ir chemiškai nepaveiktas Hiso pluošto audinys, pirmą kartą buvo ištirtas rentgenoelektroniniu mikroskopu (SEM/EDX metodu), pirmą kartą atlikta FT-IR spektroskopinė analizė ir kiti originalūs tyrimai. Pirmą kartą palyginti Hiso pluošto nefiksuotų ir formaline fiksuotų audinių mėginių tyrimų duomenys, siekiant rasti stabilius morfologinius audinių skirtumus, neišnykstančius fiksacijos buferiniame formalino tirpale metu.

Bioelektrinio impulso atsiradimui ir plitimui reikšmingi kalio, kalcio, natrio, chloro jonai, jų koncentracijos ir pasiskirstymas, todėl buvo atlikti rentgenoelektroniniai tyrimai SEM/EDX (skenuojantis elektroninis mikroskopas/energiją išsklaidanti rentgeno spektroskopija) metodu leidžiantys nustatyti tiriamos medžiagos elementinę sudėtį. Tirtuose Hiso pluošto ir skilvelių miokardo mėginiuose nustatyti fosforo, chloro, kalcio, sieros, silicio, natrio ir kalio jonų kiekiai yra vienodi. Matyt, didesnis bioimpulso sklidimo greitis Hiso pluoštu nėra susijęs su jonų koncentracijų skirtumais.

Furjė transformacijų infraraudonosios spektroskopijos (FT-IR) metodu buvo palyginti skirtingais būdais paruošti audinių mėginiai. Skirtumų tarp fiksuotų ir džiovintų Hiso pluošto ir miokardo mėginių nerasta. Skirtumai pastebėti tiriant nefiksuotus ir nedžiovintus Hiso pluošto ir miokardo mėginius. Fiksavimas buferiniame 10% formalino tirpale ir džiovinimas neturėtų paveikti stabilių, struktūrinių baltymų, todėl labiausiai tikėtina, kad rasti skirtumai tarp nevienodai paruoštų širdies audinių yra susiję su citoplazmoje esančių laisvų molekulių pokyčiais. Tokią prielaidą netiesiogiai patvirtina gauti rezultatai, rodantys, kad miokarde yra didesnis laisvų arba neigiamai įelektrintų (α-asparagino ir α-glutamo) rūgščių kiekis. Biologinių audinių FT-IR sugerties spektrai yra aminorūgščių, priklausančių baltymams ir peptidams, bei laisvų aminorūgščių spektrų suma, todėl Hiso pluošto ir miokardo mėginių FT-IR sugerties spektrų juostas priskirti konkrečių molekulinių grupių virpesiams ir įvertinti skirtumų priežastis nėra įmanoma. Negalima vienareikšmiškai teigti, kad rasti skirtumai susiję tik su laisvomis amino rūgštimis. Furjė transformacijų infraraudonosios spektroskopijos (FT-IR) metodu rasti skirtumai tarp miokardo ir Hiso pluošto audinius sudarančių biomolekulių leidžia patikimai atskirti tirtus audinius, tačiau šis metodas gali būti naudojamas tiriant tik nedidelius širdies audinio kiekius, todėl neturi didesnės praktinės reikšmės kaip vaizdinimo būdas. Be to, šis spektroskopinis tyrimas yra pakankamai sudėtingas.

Morfologinius skirtumus tarp žmogaus širdies laidžiosios sistemos Hiso pluošto ir miokardo taip pat patvirtino ir UV sugerties tyrimai. Šie tyrimai parodė skirtingus aromatinių amino rūgščių kiekius tirtuose širdies audiniuose. Trp kiekis Hiso pluošto mėginiuose buvo 4 kartus, o Tyr 2,5 karto didesnis negu MK mėginiuose. UV sugerties spektriniams tyrimams naudoti neutraliame buferiniame formaline fiksuoti ir į parafiną įlieti audiniai, todėl tikėtina, kad aromatinės amino rūgštys, lėmusios UV sugerties spektrų skirtumus yra stabilių, struktūrinių baltymų sudėtyje. Nevienodi šių aromatinių aminorūgščių kiekiai HP ir MK audiniuose gali būti susiję su skirtinga šių audinių baltymine struktūra, nuo kurios priklauso jų fiziologinės savybės. Skirtumai pastebėti trumpųjų UV-B bangų srityje (280 nm), o šio bangos ilgio spinduliuotė yra potencialiai pavojinga biologiniams audiniams, gali sukelti nudegimo reakciją, todėl praktinis šio metodo taikymas būtų sudėtingas.

Formalino tirpale fiksuotuose audiniuose nustatytus skirtumus, matyt, tikėtina rasti ir kitais, paprastesniais metodais.

Histocheminiai dažymai neišryškino didesnių Trp ir Tyr koncentracijų ŠLS. Kaip parodė UV sugerties spektrai, kasoje aromatinių amino rūgščių molekulės yra labiau polinėje aplinkoje, prieinamos vandeniui. Hiso pluošte esantį Trp supa mažesnio poliškumo aplinka, sąlygojanti spektro poslinkį į trumpųjų bangų puse, t.y. molekulės mažiau prieinamos vandeniui. Nors negalima tiksliai nustatyti priežasčių, kodėl histocheminiai dažymai neparodė didesnio aromatinių amino rūgščių kiekio širdies audiniuose, galima įtarti, kad tai lėmė skirtingas šių amino rūgščių išsidėstymas baltymuose ir skirtinga jas supanti aplinka.

Skirtumus tarp žmogaus širdies laidžiosios sistemos Hiso pluošto ir miokardo taip pat parodė ir savitosios fluorescencijos ir jos žadinimo spektroskopiniai tyrimai. Žadinant aromatinių amino rūgščių sugerties srityje (250-295 nm) pagal fluorescencijos intensyvumų ties 340 nm ir 450 nm santykius galima vienareikšmiškai atskirti HP ir MK audinius. Tačiau tokį atskyrimo metodą, kaip ir UV sugerties spektroskopiją, būtų sudėtinga taikyti *in vivo* ar rutininiams tyrimams, nes fluorescencijos žadinimui naudojama tolimosios UV-C (280-100 nm) srities spinduliuotė, kuri yra žalinga biologiniams audiniams. Aromatinių amino rūgščių sugerties srityje sužadinama plati širdies audinių savitosios fluorescencijos juosta mėlynojoje spektro srityje gali būti sąlygota keleto kitų endogeninių fluoroforų (kolageno, elastino, nikotinamidadenindinukleotido (NADH), flavinų, porfirinų, lipopigmentų ir kitų glikozaminoglikanų), sužadinamų ne tik tolimojoje UV-C (280-100 nm.) srityje, bet ir ilgesnių bangų spinduliuote. Kiekvienas fluoroforas turi savitus fluorescencijos ir jos žadinimo spektrus, jų spektrų registravimas plačiame bangų ilgių intervale leidžia išskirti atskiras juostas ir jas priskirti skirtingiems fluoroforams. Šiame darbe fluorescencija buvo žadinama plačioje spektro srityje nuo 250 nm iki 480 nm. Tokia plati žadinimo sritis sudarė galimybes selektyviai žadinti atskirus fluoroforus, surasti žadinančios spinduliuotės bangos ilgius, kuriuos būtų galima efektyviausiai panaudoti ŠLS vaizdinimui [94].

Širdies mėginių fluorescencijos ir jos žadinimo spektrų analizė leido išskirti kelis endogeninis fluoroforus su persiklojančiomis sugerties ir fluorescencijos juostomis. Rasti trys pagrindiniai fluoroforai su žadinimo/fluorescencijos spektrinėmis

juostomis ties: 280 /340 nm triptofanas, 330 / 390 nm kolagenas ir 385 / 460 nm elastinas. Skirtingi šių fluoroforų kiekiai MK, HP ir JA nulemia jų fluorescencijos spektrų skirtumus UV ir mėlynojoje spektro srityse. Labai silpną MK mėlynąją fluorescenciją galima paaiškinti žymiai mažesniais kolageno ir elastino kiekiais MK, palyginus su HP ir JA. Ypač gerai šių fluoroforų spektrų juostos išsiskiria širdies homogenatų spektruose.

Tokiais atvejais, kai endokardo jungiamojo audinio intensyvi mėlyna fluorescencija apsunkina Hiso pluošto šakų išskyrimą, fluorescenciją reikia žadinti dviem skirtingais bangų ilgiais: 330 nm (kolageno sugerties juostoje) ir 385 nm (elastino sugerties juostoje). HP ir JA audiniai skiriasi elastino fluorescencijos juostos ties 460 nm intensyvumu. JA žadinant 330 nm ir 385 nm spinduliuote šios juostos intensyvumas nekinta, o HP žadinant ties 385 nm - sumažėja du kartus. Naudojantis šiais skirtumais galima sukurti žmogaus ŠLS vaizdinimo sistemos modelį. Pagal šią vaizdinimo sistemą reikėtų keliuose endokardo taškuose registruoti dviem skirtingais bangų ilgiais sužadintus spektrus ir grafiškai atvaizduoti laidžiosios sistemo eigą. Tokiai vaizdinimo sistemai reikalinga gan sudėtinga aparatūra, o didesnės tarpskilvelinės pertvaros paviršiaus dalies taškinis skenavimas užimtų daug laiko.

Visų audinių homogenatų tirpių frakcijų spektruose dominavo Trp ir Tyr liekanų fluorescencija, o regimojoje spektro srityje fluorescuojantys endogeniniai fluoroforai liko netirpiose frakcijose. Nors visų audinių tirpių frakcijų spektrai neturi žymesnių skirtumų, elektroforezės metodu labiausiai išryškintų atskirų baltymų grupių spektrai yra skirtingi. Net artimų masių baltymai turi skirtingus fluorescencijos spektrus, tai reiškia, kad šie tirpiose frakcijose išskirti ŠLS ir MK baltymai yra skirtingų struktūrų.

Elektroforezės būdu rasta baltymų grupė (~26 kDa) aptinkama tik HP II ir III frakcijose. Pirmoje tirpioje frakcijoje, į kurią patenka mitochondrinė frakcija, citozolis ir kiti vandenyje tirpūs baltymai, ji nepastebėta. Tokiu būdu galima daryti išvadą, kad šie baltymai priklauso vandenyje netirpių membranų baltymų grupei, t.y. stabilūs struktūriniai baltymai. Rasta baltymų grupė nepriklauso žinomiems plyšinių jungčių koneksinams, kurių molekulinės masės yra 40 kDa, 43 kDa ir 45 kDa. Taip pat kitokios yra ir žinomų HCN baltymų molekulinės masės (nuo 61 kDa

iki 120 kDa). Todėl tiksliai nustatyti, kokiems baltymams priklauso Hiso pluošte rasta 26 kDa molekulinės masės baltymų grupė, šiuo metu negalima.

Pirmieji dviejų krypčių elektroforezės tyrimai taip pat parodė aiškius baltyminius skirtumus tarp žmogaus širdies laidžiosios sistemos ir miokardo. Bendras išskirtas polipeptidų kiekis buvo didesnis miokarde negu Hiso pluošte. Atskiros tirtos baltymų grupės ŠLS, skirtingai nuo miokardo, yra ne homogeniškos, o sudarytos iš dviejų baltymų grupių.

Atlikti tyrimai parodė, kad žmogaus širdies laidžiosios sistemos ir miokardo baltyminės sudėtys yra skirtingos. Kokie baltymai yra specifiški žmogaus ŠLS, kur jie išsidėstę ir kokia jų fiziologinė reikšmė bioimpulso plitimui šiuo metu negalima pasakyti. Atlikti pirmieji vaizdinimo eksperimentai parodė, kad optiniais metodais galima išryškinti ŠLS kitų širdies audinių fone. Tačiau, šiuo metu sukurtos vaizdinimo metodikos yra techniškai gana sudėtingos, todėl sunkiai pritaikomos kasdieninėje praktikoje. Detalesni proteominiai tyrimai leistų identifikuoti ir ištirti žmogaus ŠLS specifišką baltymų grupę, nustatyti šios baltymų grupės savybes ir lokalizaciją ląstelėse. Parinkus šios grupės baltymams tinkamą dažiklį arba juos pažymėjus fluorescuojančiais žymekliais, būtų galima vaizdinti širdies laidžiąją sistemą. Tokia vaizdinimo sistema būtų daug paprastesnė ir lengviau naudojama.

Išvados

1. Paruošta žmogaus širdies laidžiosios sistemos preparavimo metodika leidžianti gauti širdies laidžiosios sistemos audinį be kitų širdies audinių, ir pateikti audinių mėginius spektroskopiniams ir proteominiams tyrimams.
2. Žmogaus širdies laidžiosios sistemos ir kitų širdies audinių palyginamieji spektroskopiniai tyrimai parodė:

a. Rentgenoelektroniniu mikroskopu skirtumų tarp žmogaus HP ir MK nerasta. Fosforo, kalcio, chloro, sieros, silicio, natrio ir kalio kiekiai HP ir MK audiniuose yra vienodi.

b. Infraraudonosios sugerties spektruose yra aiškūs skirtumai tarp HP ir MK audinius sudarančių biomolekulių. FT-IR metodu atlikti tyrimai parodė, kad MK audiniuose yra didesnis laisvų arba neigiamai įelektrintų amino rūgščių kiekis.

c. UV sugerties tyrimai parodė aiškius skirtumus tarp ŠLS ir MK. Šiuos skirtumus sąlygoja nevienodi aromatinių amino rūgščių kiekiai HP ir MK.

d. Fluorescencijos ir jos žadinimo spektrų analizė parodė, kad širdies audiniuose yra trys endogeniniai fluoroforai (triptofanas, elastinas ir kolagenas) su persiklojančiomis sugerties ir fluorescencijos juostomis. Skirtingi šių fluoroforų kiekiai MK, HP ir JA nulemia jų fluorescencijos spektrų skirtumus UV ir mėlynojoje spektro srityje.

e. Hiso pluoštą ir jo šakas galima atskirti nuo jungiamojo audinio fluorescencinės spektroskopijos metodu žadinant savitąją šių audinių fluorescenciją dviejų skirtingų bangų ilgių spinduliuote: 330 nm (kolageno sugerties juostoje) bei 380 nm (elastino sugerties juostoje) ir stebint fluorescencijos pokyčius mėlynoje spektro srityje ties 460 nm. Jungiamojo audinio atveju žadinant abiejose spektro srityse fluorescencijos intensyvumas nekinta, o Hiso pluošto atveju žadinant elastino sugerties juostoje - sumažėja du kartus.

f. Širdies audinių homogenatų fluorescencijos spektrų analizė parodė, kad fluorescencija mėlynojoje spektro srityje priklauso netirpioms struktūroms. Ji stebima tik netirpių frakcijų spektruose.

3. Hiso pluošto ir skilvelių miokardo baltyminė sudėtis skirtinga. Baltymų elektroforezės metodu rasta baltymų grupė (~26 kDa) aptinkama tik HP II ir III frakcijose.

4. Rasti fluorescenciniai ir baltyminės sudėties skirtumai tarp žmogaus HP ir MK leidžia manyti, kad bioelektrinio impulso sklidimo greičių skirtumus sąlygoja ne tik ląstelių išsidėstymas, skersmuo, bet ir specifiniai morfologiniai skirtumai. Pasinaudojus nustatytais morfologiniais skirtumais galima sukurti žmogaus širdies laidžiosios sistemos vaizdinimo metodiką, kuri leistų nustatyti ŠLS anatomines ypatybes.

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