Medico Research Chronicles

ISSN No. 2394-3971

Original Research Article

CHANGES OF BLOOD PLASMA PROTEINS DURING SELECTED CARDIOVASCULAR DISEASES.

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Submitted on: September 2016
Accepted on: October 2016
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Abstract

Cardiovascular diseases are one of the most dangerous diseases in the world and that is why it is necessary constantly research and try to study these diseases not only with traditional methods but also with new techniques. This study describes laboratory methods that are applicable for the rapid early screening of cardiovascular diseases. Blood plasma of patients with thoracic aortic aneurysm, aortic valve stenosis and aortic valve regurgitation was subjected to experimental biochemical analysis by fluorescence analysis and atomic force microscopy. The structural and metabolic changes have been studied in blood plasma of patients with a thoracic aneurysm in comparison with the samples of healthy individuals. The results of fluorescence analysis showed a significant decrease of autofluorescence in blood plasma of patients in comparison with healthy subjects. Atomic force microscopy confirmed structural changes in the blood of patients in comparison with the blood of healthy subjects. The results of using new experimental laboratory methods in future could contribute to the improvement of diagnosis of selected cardiovascular diseases.

Keywords: Blood serum, cardiovascular diseases, Fluorescence spectroscopy, Atomic force microscopy

Introduction

Cardiovascular diseases belong to frequently occurring diseases of the circulatory system and present one of the main causes of mortality in the world. It is caused by different risk factors, such as heredity, smoking, obesity, diabetes mellitus, depression and others [1]. Proper prevention can help to reduce the incidence of cardiovascular diseases. During cardiovascular diseases which affect aorta (aortic aneurysm) also aortic valve (aortic valve stenosis, aortic valve regurgitation), play an important role many enzymes, proteins, growth factors, markers of inflammatory and other processes caused

degradation and remodelation of vessels and heart [2,3,4]. Study of new diagnostic methods is very important for better understanding of mechanisms of molecular-biochemical changes during diseases of heart and vessels. During diagnostics mentioned diseases very important role also plays analysis of body fluids such as blood plasma.

The goal of this work was to study the structure of blood plasma as the whole mixture (without identification of individual components of blood)by new methods which could use for characterization and early rapid diagnostics of cardiovascular diseases.

Blood and cardiovascular diseases

Blood plasma is a mixture of various proteins, enzymes, electrolytes, hormones, gases. The major plasma protein is albumin, a relatively small molecule, the principal function of which is to retain water in the bloodstream by its osmotic effect. Depletion of serum albumin permits fluid to leave the circulation and to accumulate and cause swelling of soft tissues (edema). Albumin binds certain other substances (drugs, fatty acids, hormones) that are transported in plasma and thus serves as a nonspecific carrier protein [5]. Other plasma proteins are globulins, participated in the immune system, and fibrinogen that during blood coagulation converts into fibrin [6].

Lipids are present in plasma in suspension and in solution. Blood lipids include phospholipids, free fatty acids, cholesterol and other lipids. These substances exist in plasma combined with proteins of several types as lipoproteins. The largest lipid particles in the blood, chylomicrons, consist largely of triglycerides; after absorption from the intestine, they pass through lymphatic channels and enter the bloodstream [7]. The increased level of cholesterol in the blood leads to its incorporation into the vessel wall what can cause atherosclerosis. Affected vessels change their structure; blood flow is hindered and limited. If blood flow is completed blocked, a heart attack can occur [8].

Several inorganic materials are essential constituents of plasma. Variations in the concentrations of ions in blood plasma may have profound effects on the nervous system, the muscles, and the heart, effects normally prevented by precise regulatory mechanisms.

The predominant cation of the plasma is sodium, an ion that occurs within cells at a much lower concentration. The amount of sodium in plasma is controlled by the kidneys under the influence of the hormone aldosterone, which is secreted by the adrenal gland. A diet high in sodium increases the risk of hypertension in people with sodium sensitivity, corresponding to an increase in health risks associated with hypertensions including cardiovascular disease [9].

The principal anion of plasma is chloride; sodium chloride is its major salt. Potassium, the principal intracellular cation, occurs in plasma at a much lower concentration than sodium. The renal excretion of potassium is influenced by aldosterone, which causes retention of sodium and loss of potassium. Calcium in plasma is in part bound to protein and in part ionized. Its concentration is under the control of two hormones, parathyroid hormone, and calcitonin. Vascular calcification, characterized by deposition of calcium in blood vessels during cardiovascular diseases [10], is associated with hyperphosphatemia in chronic kidney diseases. Increasing the amount of phosphorus in blood is another risk factor for cardiovascular diseases because leads to vascular calcification that increases the vascular stiffness, speed and heart frequency and decreases blood flow in coronary arteries [11]. Iron and copper are transported in plasma by metal-binding proteins (transferrin and ceruloplasmin) and are

required in trace amounts for the synthesis of essential enzymes. Iron deficiency has detrimental effects in patients with coronary artery disease and heart failure [12]. Magnesium, like potassium, is a predominantly intracellular cation and occurs in plasma in low concentration. Magnesium influences the endothelium function, inflammatory processes in the vascular wall, blood pressure [13]. Clinical hypomagnesemia and magnesium deficiency cause cardiac arrhythmia. Also, magnesium deficiency in dietary and blood plasma increases the risk of cardiovascular diseases. Magnesium decreases blood pressure, lipid levels, cardiac arrhythmia, also reduces a risk of coronary heart disease, diabetes, atherosclerosis and heart failure [13].

Materials and Methods

Experimental

The control blood plasma (n = 60) was collected by National Blood Transfusion Service in Košice from healthy blood donors. Blood plasma of experimental group (n = 60 patients with clinically diagnosed the thoracic aortic aneurysm, the aortic valve stenosis, and the aortic valve regurgitation) was provided by Department of Cardiovascular Surgery UPJŠ LF and VÚSCH, a.s. in Košice).

Members of studied groups, healthy individuals, and patients were informed by their doctor about the aim of our experimental study and they signed an informed consent. All clinical investigations were conducted according to the declaration of Helsinki principles. Ethical consent for this study has been given by the institutional committee on human research and is compliant with ethical standards on human experimentation and with the Helsinki declaration.

Material

The phosphate buffer (0.2 M; pH = 7.4) was prepared from KH_2PO_4, Na_2HPO_4 obtained from Sigma- Aldrich Chemie (Steinheim, Germany) and deionized water.

Methods

Preparation of blood plasma

The samples of blood plasma of healthy individuals and patients were centrifuged for 3 minutes at 3500 rpm (BOECO centrifuge U-32R, Hamburg, Germany), and were kept in a freezer at temperature t = - 71°C (New Brunswick Scientific, Premium U410 Ultra-Low Temperature Freezer, Enfield, Connecticut, USA).

Synchronous fluorescence fingerprint of blood plasma

The control and experimental blood plasma samples were diluted in the phosphate buffer (0.2 M; pH = 7.4) in the ratio (1:5000) were analyzed using synchronous fluorescence fingerprints on Luminescence spectrophotometer Perkin Elmer LS 55 (Waltham, Massachusetts, USA) at room temperature 25°C. The fluorescence measurements were performed at excitation wavelength fromλ = 200 to 400 nm. Individual measurements were processed into contour and three-dimensional synchronous fluorescence fingerprints (SFF) using the software WinLab (Perkin Elmer, Waltham, Massachusetts, USA). Synchronous simple excitation spectra of blood plasma samples were the result of the horizontal cut of SFF at $\Delta\lambda$ = 50 nm. These simple fluorescence spectra were selected as a simple model for comparison spectra of blood plasma of healthy subjects and patients with cardiovascular disease.

Atomic force microscopy of blood plasma

The samples of control and experimental blood plasma (5µl) layers deposited on the glass slides of patients and control group were analyzed using an atomic force microscope Dimension Icon® (ICON, Bruker, Berkley, California, USA) in tapping mode with silicon tips (Mikro Masch, Berkley, California, USA, NSC35 series) with radius of curvature ~ 10 nm. The surface of each sample was processed by the software Scan Asyst™ and dried at room temperature.

Statistical analysis

The values of fluorescence intensity of control and experimental samples were statistically compared using Student – Newman – Keuls Multiple Comparisons Test.

Results and Discussion

Fluorescence spectroscopy analyses various metabolic and pathologic changes in cells, tissues and biological fluids. This method is one of the most sensitive analyses that are used for monitoring of fluorescence intensities of fluorophores. Blood plasma presents a mixture of various endogenous fluorophores (aromatic amino acids, proteins, and coenzymes) participated on metabolic pathways. The main fluorophores in proteins are aromatic amino acids tryptophan, tyrosine, phenylalanine with fluorescence excitation and emission maxima, Trp (λ_{ex} = 295 nm/ λ_{em} = 353 nm), Tyr (λ_{ex} = 275 nm/ λ_{em} =304 nm), Phe (λ_{ex} = 260 nm/ λ_{em} = 282 nm).

Blood plasma of patients with a thoracic aortic aneurysm at λ_{max} = 280 nm (proteins excitation)showed statistically decrease of the autofluorescence ($p<0.001$) o 67 ± 0.11 % in comparison with blood plasma of healthy individuals (figure 1). Fluorescence excitation maxima of characteristic proteins (albumin, globulin, fibrinogen) in blood plasma were observed at λ_{ex} = 280 nm.

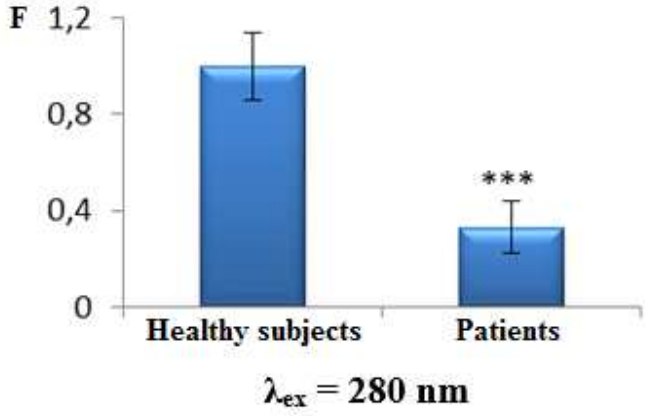

Figure 1 Statistical comparison of the autofluorescence of blood plasma of patients (n=60)and control group (n=60)at λ_{ex} = 280 nm.

The damaged tricuspid valve (figure 2, F = 340) as well as damaged bicuspid valve (figure 2, F = 321) were diagnosed in patients with thoracic aortic aneurysm. In this characteristic group of selected patients was observed the decrease of the fluorescence intensity (figure 2) in comparison with healthy individuals (F = 700). The fluorescence intensity was higher (figure 2) in patients with aortic regurgitation and with the damage bicuspid valve (F = 321) in comparison with patients who suffered from aortic valve stenosis and the damage bicuspid valve (F = 167) and tricuspid valve (F = 140).

Simple synchronous excitation spectra were created by a horizontal section of SFF blood plasma at $\Delta\lambda$ = 50 nm, where was observed the differences of the experimental samples of blood plasma in comparison with control group (figure 2). Synchronous excitation spectra graphically showed characteristic complex composition and changes of blood plasma healthy individuals and selected patients with thoracic aortic aneurysm. The greater

decrease in the fluorescence intensity at λ_{ex} = 280 nm was detected in patients with aortic valve stenosis and with damaged tricuspid valve (F = 138) than in patients with a damaged tricuspid valve (F = 167). In patients with thoracic aortic aneurysm was diagnosed either damaged tricuspid valve (F = 350) or bicuspid valve (F = 320). In this group of selected patients was observed at λ_{ex} = 280 nm decrease of fluorescence intensity in comparison with healthy subjects (F = 702). The fluorescence intensity was higher in patients with aortic regurgitation and with damaged bicuspid valve in comparison with patients with aortic valve stenosis and damaged bicuspid valve (F = 167) eventually tricuspid valve (F = 138).

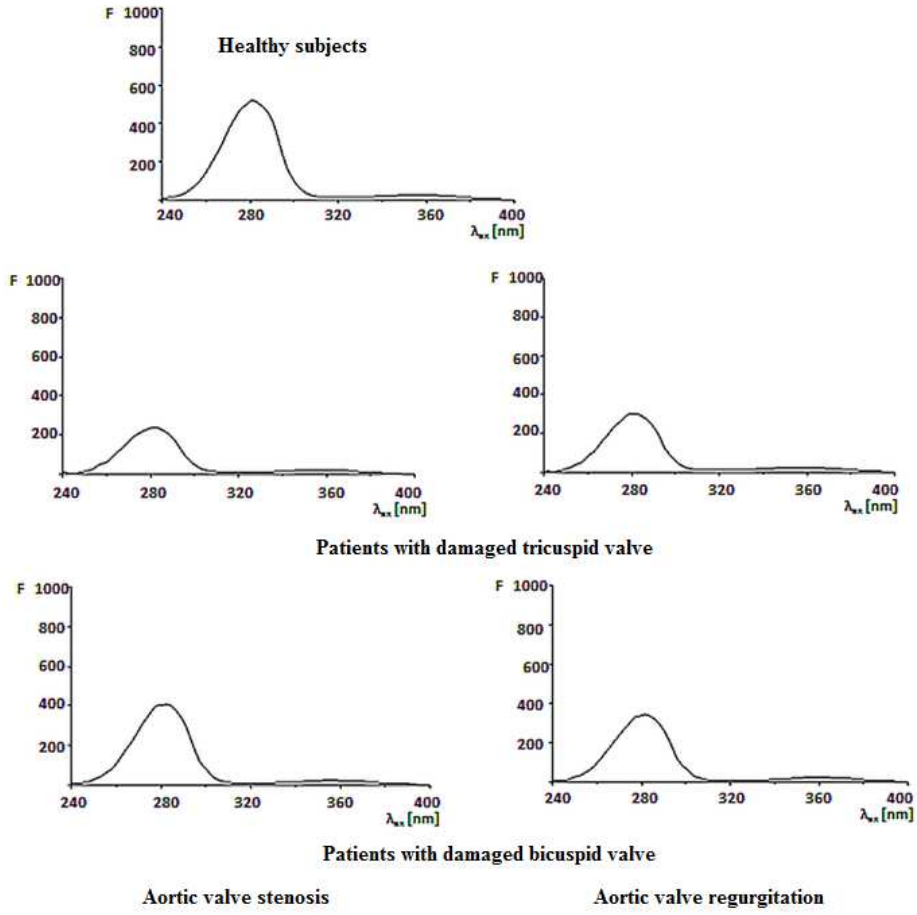

Figure 2 Simple synchronous fluorescence excitation spectra ($_{\Delta}\lambda$ = 50 nm) of blood plasma of representative control group of healthy subjects and selected representative patients with thoracic aortic aneurysm and other selected cardiovascular diseases: aortic valve stenosis (left) and aortic valve regurgitation (right).

Contour synchronous fingerprints are shown in figure 3. In experimental (n = 60) and control group (n = 60), there is one fluorescence centrum that is located at $\Delta\lambda$ = 70 nm/278 nm, but higher fluorescence intensity in healthy subjects (F = 700) than in patients with selected cardiovascular diseases. An extended shift of higher fluorescence intensity of contours towards higher wavelengths in range $\Delta\lambda$ = 40-50

nm/325-330 nm was detected at contour fluorescence maps of patients with aortic valve stenosis (figure 3) in comparison with healthy individuals and patients with aortic valve regurgitation. In patients with diagnosed aortic valve stenosis were observed several biochemical changes affecting the aortic valve structure: increased accumulation of inflammatory cells (macrophages, T lymphocytes) and apolipoproteins (apoA, apoB, and apoE), the process of calcification on leafs of valve [14, 15].

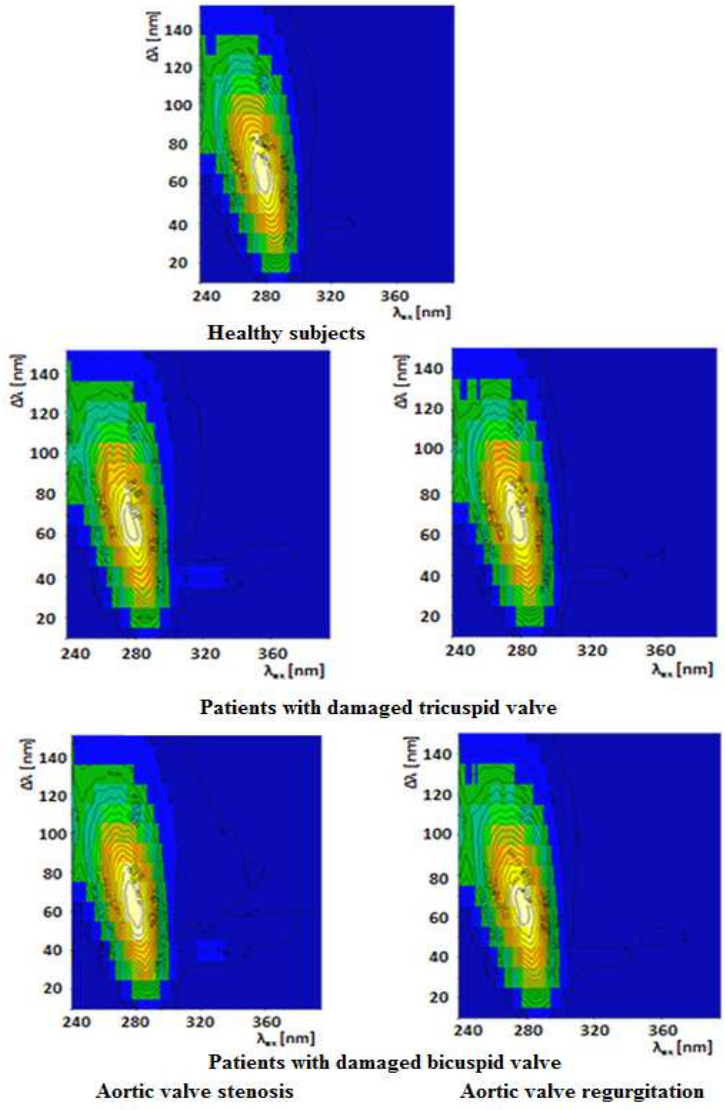

Figure 3 Contour synchronous fluorescence fingerprints of blood plasma of representative characteristic control group of healthy subjects and selected representative patients with thoracic aortic aneurysm and other selected cardiovascular diseases: aortic valve stenosis (left) and aortic valve regurgitation (right).

The changes of protein structure were detected on surfaces of blood plasma samples using atomic force microscopy. This method that allows a three-dimensional view and detection of various surface structures are based on a movement of the small tip on studied surface[16].In recent

years AFM is used in research and study of cardiovascular diseases [17, 18].

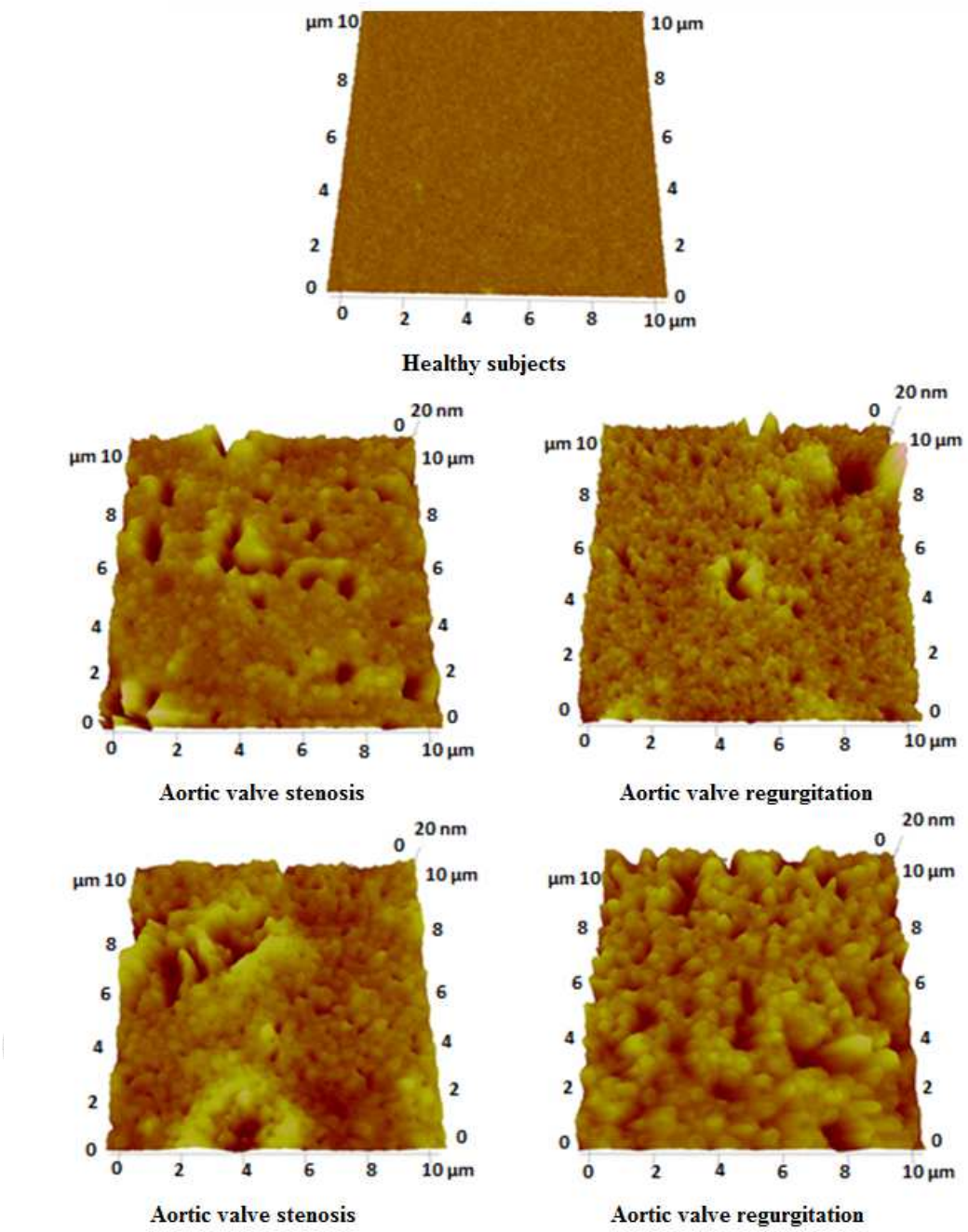

Figure 4 Blood plasma samples of the control group and patients with thoracic aortic aneurysm and other selected cardiovascular diseases: aortic valve stenosis (left) and aortic valve regurgitation (right) studied by atomic force microscopy.

On the surface of blood plasma samples of patients with thoracic aortic aneurysm and aortic valve stenosis, there were detected well-defined circular 400 – 500 nm large globules that caused higher roughness (rq = 8,64 - 12 nm) of these samples in comparison with control group (rq = 1,35 nm). A control group of blood plasma showed small 30 nm globules (figure 4). Blood plasma samples of patients with thoracic aortic aneurysm and aortic valve regurgitation showed higher roughness (rq =

4, 4 nm - 9, 24 nm) caused by the presence of fibrils (400 nm) and regular globules (300 nm) in comparison with control group (figure 4).

The differences in blood plasma of patients in comparison with healthy subjects indicate that during studied cardiovascular diseases are observed structural changes of individual components (proteins, lipids, saccharides) in studied biological fluids. Proteolytic enzymes which participate on the degradation of structural components in the aortic wall increase the risk of an aortic aneurysm. This cardiovascular disease leads to increased production of matrix metalloproteinases (MMP-2, MMP-9, and MMP-12). These major proteases cause the degradation collagen and elastin fibers, following structural changes in tissue and remodeling of the vessel wall, what leads to an aortic aneurysm [19].

Conclusion

This work described the biochemical composition of the blood plasma, clinical and biochemical methods of sampling and proteomic analysis, their options and limitations of usage. The comparison of autofluorescence and surface of proteins of the blood plasma of healthy subjects with blood plasma of patients with cardiovascular disease diseases (with clinically diagnosed thoracic aortic aneurysm, the aortic valve stenosis and the aortic valve regurgitation)are a rapid and sensitive analysis of changes of total blood plasma proteins from one drop (1 - 5μl) of blood plasma. Results of fluorescence analysis showed a significant decrease of autofluorescence of blood plasma ($p < 0.001$) of patients in comparison with healthy subjects at λ_{ex} = 280 nm where are localized characteristic excitation maxima of aromatic amino acids of proteins. Atomic force microscopy revealed changes of blood plasma structure of patients in comparison with healthy subjects. In the blood of patients, there were observed greater globules, fibrils, and higher roughness. Fluorescence spectroscopy and atomic force microscopy has not been used yet for clinical investigations of blood plasma but could be new tools in the early, sensitive and rapid diagnosis of heart pathologies and other diseases.

Acknowledgements

This work was supported by the Slovak Academy of Science and the Slovak Grant Agency for Science under the contract No. *VEGA-1/0115/14*.

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