A study of fluorescence spectra of peripheral arteries: intrinsic fluorophore emission in the intima under hypodynamic stress

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We have examined the effects of hypodynamic stress (as one of the risk factors for cardiovascular disease) on the central ear arteries on rabbits and associated arterial injury. A 48-day hypodynamic stress was provoked in Chinchilla male rabbits by confining them to a confined space with a limited potential for movement. The intima of the central ear arteries was examined by autofluorescence and electron microscopy. Laser-induced fluorescence was examined during excitation at a range of wavelengths 320–360 mm at the absorption maxima of several fluorescing connective tissue proteins. Changes in fluorescence profiles of an intact artery and an artery affected by hypodynamic stress were detected in the spectral range from 370 to 550 nm. Spectroscopic examination revealed a decreased relative fluorescence intensity at around 410 nm and red-shifted fluorescence emission for affected artery wall in comparison with intact artery intima. Ultrastructural studies indicated that the internal elastic lamina and subendothelial layer were disintegrated or lessening, and cholesterol had accumulated in the inner wall of central ear arteries in stress-affected rabbits. Initial results show that structural changes of the central ear artery intima due to hypodynamic stress can be monitored using fluorescence spectroscopy.

Keywords: autofluorescence, artery, ultrastructure, hypodynamics

INTRODUCTION

Atherosclerosis as a disease is realized through structural (accumulation of lipids, proliferation of smooth muscle cells and production of connective tissue in the intima) and functional (impaired vasodilation components of the involved artery) changes [1]. Endothelial cells damage the interface with the main risk factors of atherosclerosis. Thus, the influence of various risk factors can be explained by the approach to atherosclerosis as an injury reaction. Commonly, a light arterial injury occurs, causing only cell functional disorders – so-called endothelial dysfunction. Under a long-term injury, the reparation process is irreversibly damaged, thus, the permeability for plasma components and lipids at damaged endothelial sites increases. Therefore, subtle biochemical changes in the intima gradually transform into structural changes of the arterial wall (atheroma). Conventional diagnostic methods of vascular disease have some weakness in defining the prime undistinguished alterations *in situ* [2]. In principle, there is no available technique in cardiovascular medicine capable to image early stage lesions and prevent their progression. Thus, the techniques for the evaluation of functional-structural disorders in the arteries associated with early stages of atherosclerosis are important for the diagnostics and correction purposes.

According to the WHO and the International Society and Federation of Cardiology, physical inactivity is an important risk factor for developing coronary heart disease [3]. It has been confirmed that physical inactivity contributes to population risk more than hypertension, elevated cholesterol levels or obesity. In tissues, physical inactivity provokes changes in the concentration of bioelements that participate in the metabolism of lipids, carbohydrates, secretion of hormones, stability of membranes and contractility of cardiomyocytes [4, 5]. All this allows assuming the possible early injuries in blood vessels and the importance of detecting these structural changes caused by physical inactivity. The biochemical changes in the arteries, caused by physical inactivity, have not yet been studied by fluorescence spectroscopy. As there is a close functional connection between coronary and peripheral arteries [6], the latter are expedient to use for monitoring the development of disease.

Autofluorescence spectroscopy has been investigated as an effective semi- or non-invasive tool for sensing

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changes in the morphology and biochemistry of tissues associated with disease development [7, 8]. Currently there is considerable interest in the use of fluorescence spectroscopy for discriminating between the lesion and the surrounding normal tissue. Both the intensity and line shape of the fluorescence spectrum play a significant role in evaluating the distribution of native fluorophores in the tissue and improving discrimination specificity by induced optical contrast [7, 9–12]. Fluorescence spectroscopy has been employed not only to study the biochemical basis of the normal and atherosclerotic arterial wall, but also to identify atheroma or to detect triggered plaque disruption and trombosis [13–16]. Different models of *in vivo* and *in vitro* systems of human and animal tissues have been studied to determine the chemical constituents responsible for the spectra to provide the method for tracking the development of disease *in vivo* [17–20]. The observed difference in the arterial tissues' fluorescence profile was largely ascribed to the contributions of the structural proteins, elastin and collagen, as the main sources of autofluorescence in arterial tissue [13, 21]. At the red-shifted wavelengths, the contribution of the emission from lipid components which are typical of macrophages and lipid-rich lesions was also reported [16].

In this study, the possibility to inflict hypodynamic stress on rabbits and to detect structural disorders in the intima of central ear arteries using fluorescence spectroscopy was investigated. Data on hypodynamic stress injury level in the arterial intima were obtained by ultrastructural examination with an electron microscope and attributed to differences in fluorophore expressivity that contributed to arterial tissue autofluorescence.

MATERIALS AND METHODS

Hypodynamic stress of 48 days duration was provoked according to B. M. Fiodorov [22] in Chinchilla male rabbits (weight 2.5–3.0 kg) by placing them in metal hutches and subjecting to the rigors of a confined space. The control group rabbits had no intervention and were kept in vivarium conditions. After 48 days of hypodynamic regimen, the rabbits were put to sleep using a lethal dose of thiopental-sodium solution (35 g/kg). Specimens of central ear arteries were subsequently dissected from the ear. The tissue specimens were taken from animals subjected to hypodynamic stress (n = 5) and from the control group (n = 5). Two central ear arterial specimens (each 2 cm in length) were taken from each animal – one specimen for fluorescence spectroscopic analysis and one specimen for ultrastructural analysis.

Specimens for morphological analysis were fixed in the solution containing 2% of paraformaldehyde and 2.5% of glutaraldehyde in 0.1 mmol/l cacodylate buffer (pH 7.4) for more than 4 h at room temperature or overnight at 4 °C. The specimens were then post-fixed for 2 h with 1% of osmium tetraoxide solution in 0.1 mmol/l cacodylate buffer (pH 7.4) dehydrated through a graded ethanol series and embedded in Epon 812 and Araldit mixture. Ultrathin sections stained with uranyl acetate and lead citrate were evaluated by electron microscopy (Philips-300).

After arterial specimens' dissection and before fluorescence measurements, the specimens were kept in Ringer solution (Balkanpharma) for less than 1 h. Tissue fluorescence emission was induced using tunable radiation of Topas optical parametric generator (Ligth Conversion Ltd.) pumped by 2nd harmonic of a picosecond Nd:glass laser (Ligth Conversion Ltd.) for excitation (Fig. 1). On the output of the parametric generator, 1ps pulses were provided with an energy of up to 36 μJ per pulse at a typical laser repetition rate 20 Hz. Laser-induced fluorescence was examined during excitation in the range of excitation wavelengths 320–360 nm with an increment of 5 nm. The laser beam was focused onto the specimen surface (spot size 1 mm^2) located on a nonfluorescing mount. The fluorescence light was collected with an optical fiber fixed perpendicularly to the specimen surface at a distance of 1–2 mm to view only the specimen area illuminated by the laser beam (spectral acquisition was carried out with a 9 s laser exposure time). Fluorescence spectra were recorded using a flat-field grating polychromator (S380 Solar TII, Ltd.) with a cryogenically cooled OMA-IV CCD camera (EG&G Instruments Corp.).
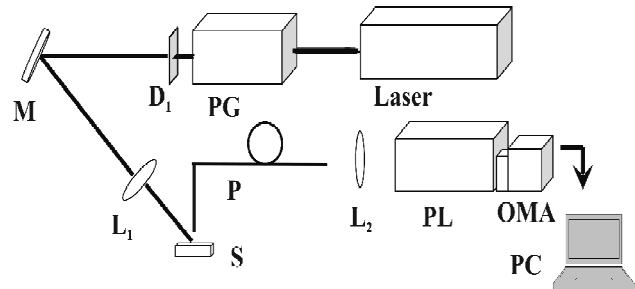

Fig. 1. Experimental setup. Laser – picoseconds Nd:glass laser, PG – parametric generator, D1 – dichroic mirror, M – mirror, L1 – quartz lens, S – sample folder, P – fiber optic probe, L2 – objective, OMA – optical multichannel analyzer, PC – computer

The specimens were open longitudinally and located on a nonfluorescing mount facing the intima surface to the laser beam. The sample mount allowed to move horizontally to excite a different place of the intima each time so that spectra from the whole area were obtained. We analyzed five ear arteries from five different control rabbits and five arteries from five different rabbits subjected to hypodynamic stress. The recorded spectra are the sum of up to 5 individual spectra normalized to unit area after background subtraction and correction for the spectral response of the system. During measurements, the specimens were moistened with Ringer solution.
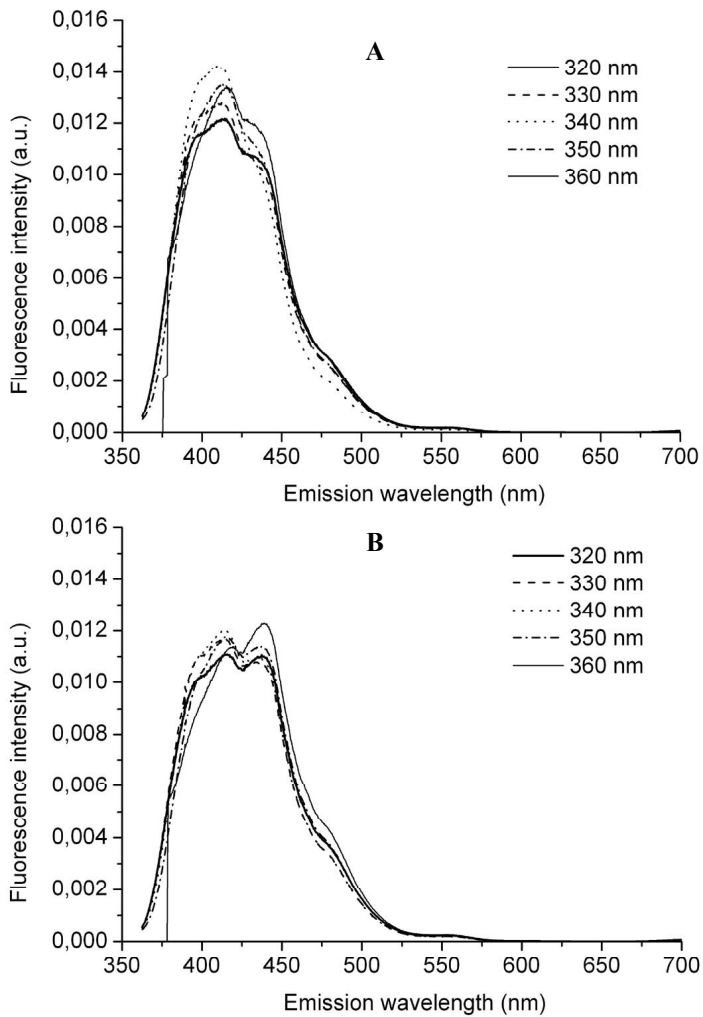
Fig. 2. Mean autofluorescence spectra of the intima of intact artery (A) and artery affected by hypodynamic stress (B) under different excitation wavelengths. Fluorescence spectra are normalized to unit area

RESULTS AND DISCUSSION

The autofluorescence spectra of intact and hypodynamic stress affected arterial wall intima were recorded for 10 different arterial specimens exciting at different wavelengths (320–360 nm), and the averaged spectra are presented in Fig. 2 A, B. Using selective excitation by tunable radiation, the autofluorescence spectra recorded from the internal surface of rabbit central ear artery showed significant differences in relative fluorescence emission intensity and fluorescence emission spectral shapes within a spectral range of 370–520 nm. The valley between the two peaks in the fluorescence emission spectra is due to hemoglobin fluorescence reabsorption and is centered at around 420 nm. Two bands with peaks at around 410 and 445 nm can be distinguished in intact and affected arterial specimens. A slight broadening to the longer wavelengths (450–550 nm) was characteristic only of affected vessel wall. A marked decrease in the normalized fluorescence emission intensity at 410 nm was observed for affected vessel wall (fluorescence intensity at 410 nm dropped by about 1.3)

exciting at 340 nm. The corresponding autofluorescence excitation–emission matrices (EEMs) for intact and hypodynamic stress affected arterial specimens are displayed in Fig. 3 A and B, respectively. The shaded scale represents different fluorescence intensities. In case of autofluorescence EEM for intact central ear artery wall (Fig. 3 A), the dominant fluorescence peak was observed near 410 nm, and the maximum relative fluorescence intensity was reached for excitation at 340 nm and emission around 410 nm, whereas in EEM for affected vessel wall a two-fluorescence-band structure was more pronounced (peaks around 410 and 445 nm) under selected fluorescence excitation wavelengths. As follows from differential EEM (Fig. 4), the most noticeable differences in spectral shapes of intact and affected internal artery wall specimens were observed for excitation at 340 nm and 355 nm. The relative fluorescence intensity decreased near 410 nm, but it remained the same at around 445 nm for affected artery intima when excited at 355 and 360 nm, while for intact artery we observed only a variation in relative emission intensity near 410 nm exciting from 320 to 360 nm. The spectroscopic examination indicated characteristic differences in intact and affected artery emission spectra, because the different expressivity of intrinsic fluorescent compounds is related to a 48-day subjection to confined space.

According to previous works, there are at least two fluorescent components responsible for autofluorescence in most blood vessels [13, 15]. These fluorophores are collagen and elastin which are associated with matrix structure. Nearly all vessels on the arterial side of the circulation have an internal elastic lamina which lies between the endothelium and the innermost layer of smooth muscle cells and is responsible for the elasticity of the vessel. Elastin is largely found in the media and internal elastic lamina. Most of the extracellular material between the smooth muscle and in the adventitia layer is collagen which is also autofluorescent. Only larger vessels can produce significant fluorescence from bundles of collagen fibers. Upon UV radiation for fluorescence excitation, elastin exhibits an about twice broader emission spectrum with the peak centered at around 410 nm than does the collagen emission which is blue-shifted and peaking at around 390 nm [23]. Thus, it is likely that relative emission intensity from elastin and collagen was more intense in the control (intact) than in the affected tissue. EEM analysis of the spectra recorded from rabbit artery inner wall surface *ex vivo* demonstrated clearly delineated changes in relative fluorescence emission intensity and a spectral shape associated with vascular injury under hypodynamic stress.

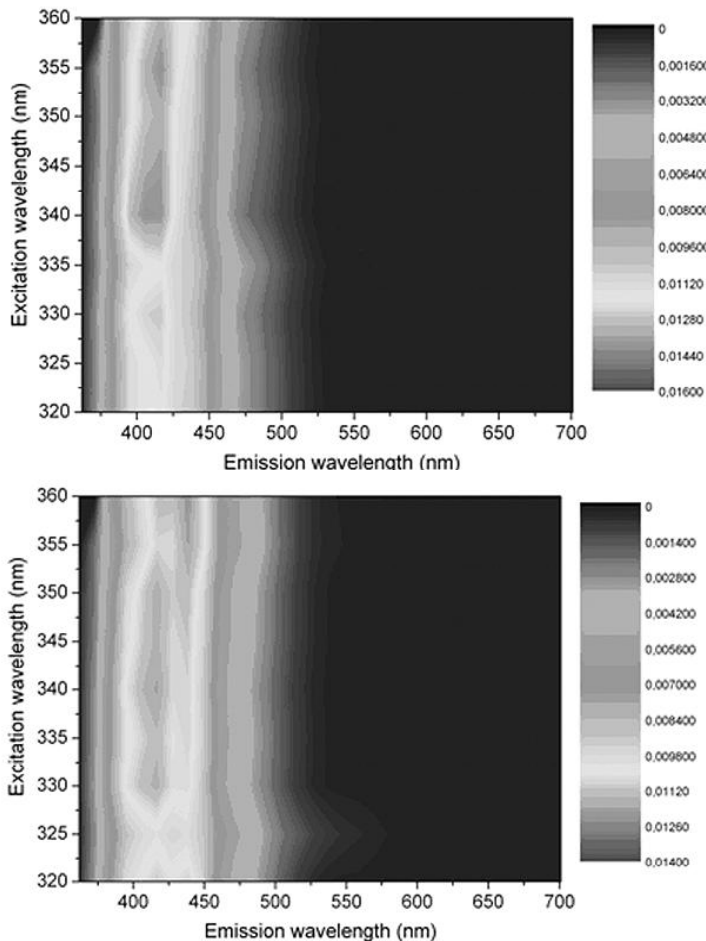The observed differences might be related to a decrease of elastin. Ultrastructural examination revealed

Fig. 3. Representative excitation – emission matrices (EEMs) of the intima of intact artery (A) and artery affected by hypodynamic stress (B). Each spectrum is normalized to unit area

that affected rabbit arterial specimens exhibited alterations of the vessel wall structure: lessening or disappearance of the subendothelial layer consisting of elastin and collagen, and disintegration of internal elastic lamina (Fig. 5 B, C). In the ultrastructure of the intact artery wall (Fig. 5 A) we observed clearly delineated elastic lamina and a subendothelial layer. The presumable interpretation of these observations is that the

spectroscopic differences at around 410 nm between the intact and the affected vessel walls are due to a change in the relative amount of elastin. Since the strong fluorescence of collagen (emission peak at 390 nm) is related to an advanced atherosclerotic process [24, 25], our data might be indicative of an early arterial wall injury under hypodynamics.

We found that the fluorescence spectrum recorded from an affected artery wall showed a broader curve slope to the longer wavelength region. This may be related to incorporation of lipids in the intima of affected arteries (Fig. 5, C). According to the literature, certain lipids that accumulate in arterial wall during development of atherosclerosis have been shown to emit red-shifted fluorescence [26, 27]. These fluorescent lipids include the lipopigment ceroid, free cholesterol, and cholesterol esters.

In order to determine the spectral regions presenting significant fingerprints for the changes in the structure and content of tissue fluorophores, we calculated differential autofluorescence EEM (Fig. 6) by subtracting the EEM of an affected artery from the EEM of an intact artery wall. The positive differential EEM region in-dicates a decrease of relative elastin contribution to total autofluorescence, thus, the negative EEM region indicates the increased input of lipid components.

CONCLUSIONS

We performed laser-induced fluorescence spectroscopy experiments in Chinchilla male rabbits and found that morphological changes of the peripheral artery intima induced by hypodynamic stress can be monitored using the information derived from tissue autofluorescence. The collected spectroscopic results were contrasted against ultrastructural studies of the arterial wall by electron microscopy. Differences in the developmental arterial in-
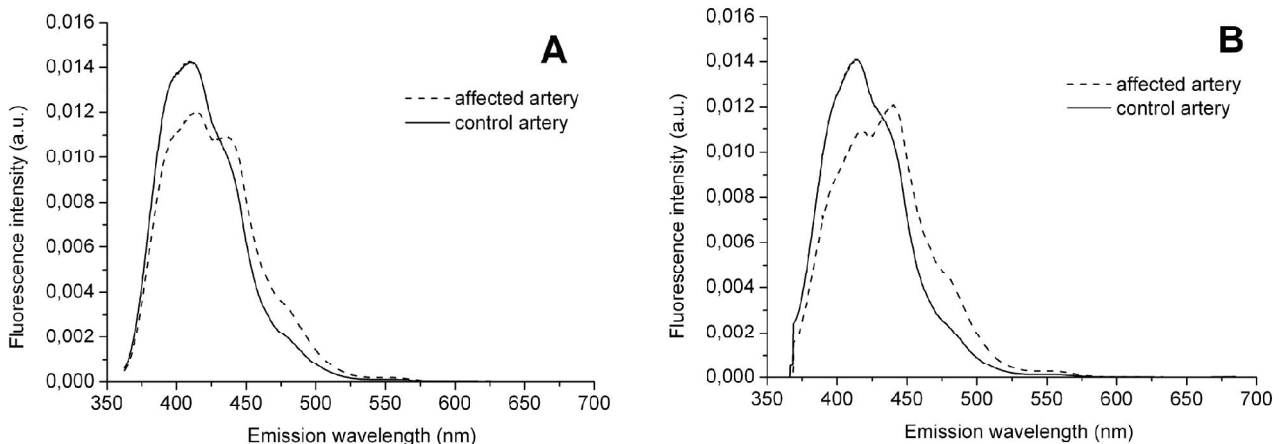

Fig. 4. Mean autofluorescence spectra of the intima of intact arteries and those affected by hypodynamic stress for 340 nm (A) and 355 nm (B) excitation wavelength
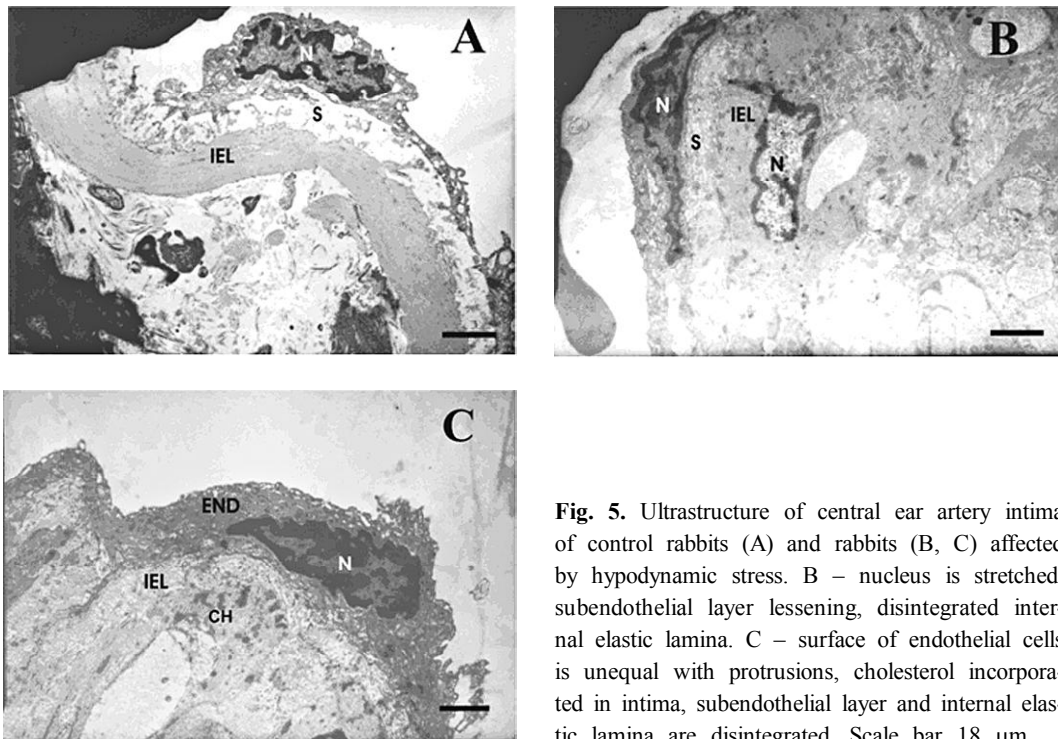
Fig. 5. Ultrastructure of central ear artery intima of control rabbits (A) and rabbits (B, C) affected by hypodynamic stress. B – nucleus is stretched, subendothelial layer lessening, disintegrated internal elastic lamina. C – surface of endothelial cells is unequal with protrusions, cholesterol incorporated in intima, subendothelial layer and internal elastic lamina are disintegrated. Scale bar 18 μm
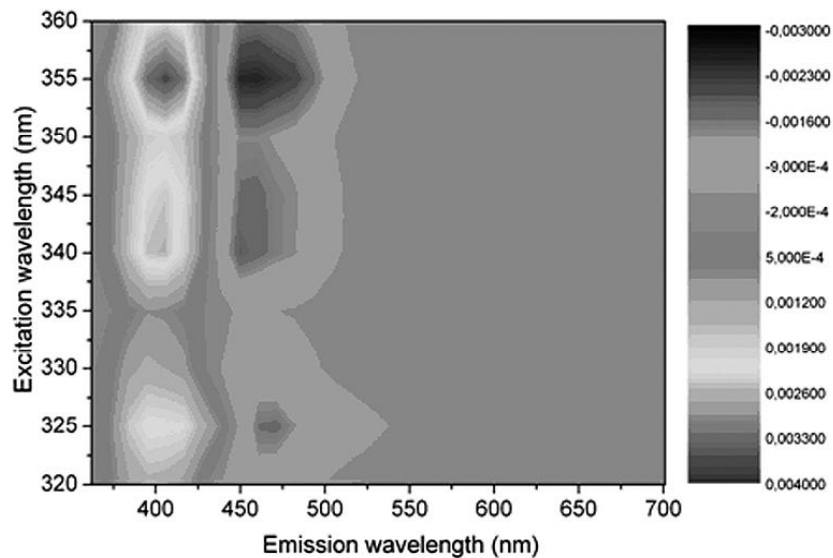

Fig. 6. Differential 2-dimensional plot of *ex vivo* excitation-emission matrices (EEMs) constructed by subtracting stressed artery intima EEM from intact artery intima EEM

jury are believed to contribute to the changes in autofluorescence spectra recorded at 48 days following subjection to a confined space. The most probable interpretation of these findings is that the spectroscopic differences in the blue spectral region between intact and affected arterial walls is due to a change in the relative occurrence of elastin and collagen, while red-shifted fluorescence corresponded to lipid components present in the affected artery. The differential excitation–emission matrix of simple construction might reveal the spectral regions most informative for an early detection of artery injuries caused by hypodynamic stress.

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PERIFERINIŲ KRAUJAGYLIŲ FLUORESCENCIJOS SPEKTRŲ TYRIMAS

S a n t r a u k a

Šiame darbe fluorescencinės spektroskopijos metodu buvo tirtas hipodinaminio streso poveikis periferinėms kraujagyslėms. Iš šinšilos veislės triušių (paveiktų hipodinaminio streso ir kontrolinės grupės) centrinių ausies arterijų paimti bandiniai, užregistruoti jų savosios fluorescencijos spektrai. Taikant atrankinį žadinimą derinamo bangos ilgio spinduliuote, didžiausi skirtumai tarp sveikos ir pažeistos kraujagyslės sienelės stebimi (370–550 nm) spektriniame intervale. Santykinio fluorescencijos intensyvumo sumažėjimas ties 410 nm, taip pat platesnis šlaitas į ilgabangę pusę buvo charakteringi pažeistai kraujagyslei, lyginant su sveikąja. Pažeistos arterijos intimos ultrastruktūros tyrimai elektronų mikroskopu rodo, kad vidinė elastinė membrana ir subendotelinis sluoksnis yra fragmentuoti, vietomis susiaurėję, kai kur matyti susikaupęs cholesterolis. Periferinių kraujagyslių sienelių 370–550 nm spektriniame ruože fluorescuoja keli fluoroforai, kurių fluorescencijos spektrai persikloja. Manome, kad spektrinius pokyčius mėlynoje spektro srityje lemia elastino ir kolageno santykinio kiekio pokytis, tuo tarpu spektro poslinkį į ilgabangę pusę – lipidai. Mūsų tyrimo duomenimis, hipodinaminio streso sukelti morfologiniai pokyčiai periferinių kraujagyslių intimoje gali būti atpažinti remiantis autofluorescencijos spektrų analize.