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# Recognition by laser-induced fluorescence spectroscopy *ex vivo* of structural changes in thoracic aorta intima of rabbits subjected to hypodynamic stress

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The investigation was performed to evaluate the possibilities of fluorescence spectroscopy in recognising structural changes of the thoracic aorta intima in rabbits subjected to hypodynamics (as a risk factor for cardiovascular disease).

A 48-day hypodynamic stress was provoked in Chinchilla male rabbits (n = 8) by placing them in metal hutches. The control (n = 8) rabbits had no intervention and were kept under normal vivarium conditions. The ultrastructure of thoracic aorta intima samples (of each control and each subjected to hypodynamics rabbits) was evaluated by electron microscopy (Philips-300). Also, fluorescence spectra characteristics of each control aorta intima and each aorta intima affected by hypodynamic stress at varying excitation wavelengths (340–415 nm) were recorded by picosecond Nd laser spectroscopy in the spectral range of 375–675 nm. An increase in the intensity of fluorescence emission in the stressed aorta intima at a wavelength close to 570 nm (excitation 345–365 nm) and a lowered fluorescence emission intensity at around 445 nm (excitation 350–365 nm) were determined in comparison with the control aorta intima ($P < 0.05$). Fluorescence excitation-emission matrices also showed a more intense fluorescence for the control aorta intima at a wavelength of around 445 nm, while expressive fluorescence was shown by the stressed aorta at longer wavelenghts (around 570 nm). At the same time, cholesterol was accumulated in the inner wall of the thoracic aorta, the elastica interna and the subendothelial layer were disintegrated in stress-affected rabbits. Initial results of laser fluorescence spectroscopy promise the possibility of discriminating between normal and abnormal aorta intima based on ultrastructural alterations caused by hypodynamic stress.

**Key words:** fluorescence, hypodynamic stress, thoracic aorta, ultrastructure

## INTRODUCTION

The main factors that stimulate physical inactivity are the consequences of the industrial revolution, i.e. elimination of hard physical work, and low energy input in the working activity. New technologies (television, computer communications, electronics in workplaces, computer games, the Internet, etc.) significantly decreased people's physical activeness in their professional activity and during their leisure time [1]. Sedentary lifestyle and smoking have been identified by McGinnis & Foege [2] as the most important causes of death in the American population. In addition to that, sedentary lifestyle is a major risk factor for cardiovascular diseases comparable to hypercholesterolemia, hypertension, and cigarette smoking [3]. According to the WHO and the International Society and Federation of Cardiology, physical inactivity is a risk factor for coronary heart disease [4]. Thus, in case of hypodynamics, it is important to detect early damage to the ultrastructure of blood-vessels. Besides, evaluation of early structural disorders of blood vessels that are coronarographycally unseen is important for diagnostics and correction. Laser-induced fluorescence spectroscopy in particular may prove useful in the early detection of diseased tissue. This relies on the fact that tissue chromophore (elastin, collagen, lipids, etc.) content and their fluorescence intensity vary depending on the state of the disease [5]. Fluorescence emission analysis can detect and differentiate collagen and elastin components in the arterial wall [6] and cardiac valve leaflets [7, 8]. Thus, for the solution of the above-mentioned problem, first of all it is necessary to define the relationship between the intensity of fluorescence of endogenous fluorophores und ultrastructural damage inflicted by hypodynamics in blood vessels. All this would allow

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for the identification of damage to the blood vessel intima by applying fluorescence spectroscopy. Thus, the aim of this study was evaluation of the possibility to recognize the structural damage to the intima of thoracic aorta under hypodynamic stress by applying selective excitation of tissue-endogenous fluorophores by tunable radiation.

## MATERIALS AND METHODS

### Induction of hypodynamic stress

The animals were cared, used and killed according to the rules defined by the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purpose (Licence No. 61/2004). Hypodynamic stress of 48 days in duration was induced according to Fiodorov [9] in Chinchilla male rabbits (weight 2.5-3.0 kg, n = 8) by placing them in metal hutches that tightly enclosed their bodies. The rabbits, however, could freely feed and drink. The control rabbits (n = 8) had no intervention and were kept in vivarium conditions.

### Procedures of thoracic aorta specimen dissection

After 48 days of hypodynamic regimen, the rabbits were put to sleep using a lethal dose of thiopental-sodium solution (35 mg/kg). On opening the chest, samples of thoracic aorta of experimental (n = 8) and control (n = 8) rabbits were excised to determine the ultrastructure and fluorescence of endogenous fluorophores in the intima of the aorta. Two thoracic aorta specimens (each 2 cm in length) were taken from each animal – one specimen for fluorescence spectroscopic analysis and one specimen for ultrastructural analysis. After dissection and before fluorescence measurements, the specimens were kept in Ringer solution (Balkanpharma) for less than 1 h.

### Ultrastructural studies

Thoracic aorta specimens for morphological analysis were immersed into a fixative 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. Thoracic aorta samples were then post-fixed for 2 h with a 1% 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).

### Laser-induced fluorescence spectroscopy instrumentation

Tissue fluorescence emission was induced using tunable radiation of a Topas optical parametric generator (Ligth Conversion Ltd.) pumped by the 2nd harmonic of a picosecond Nd glass laser (Light Conversion Ltd.) for excitation (Fig. 1). On the output of the parametric generator, 1 ps pulses were provided with an energy of up to 36 μJ per pulse at a typical laser repetition rate 20 Hz.

### Measurements of laser induced fluorescence

Laser-induced fluorescence was examined during excitation in the range of excitation wavelengths 340–414 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). 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 analysed eight thoracic aortas from eight different control rabbits and eight thoracic aortas from eight

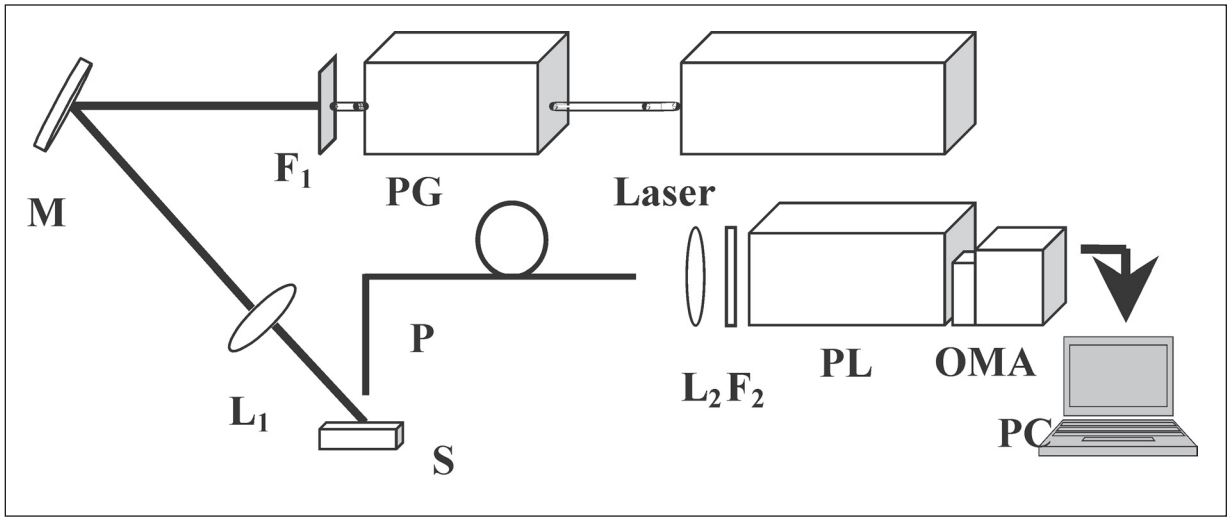


**Fig. 1.** Experimental setup. Laser – picoseconds Nd:glass laser; PG – parametric generator; $F_1$ – excitation filter set; M – mirror; $L_1$ – quartz lens; S – sample folder; P – fiber optic probe; $L_2$ – objective; $F_2$ – emission filter set; OMA – optical multichannel analyser; PC – computer

Table. **Effects of hypodynamic stress on the intensity of fluorescence**

| Excitation wavelength | Emission wavelength | | |
|---|---|---|---|
| | 445 nm | 470 nm | 570 nm |
| 340 nm | | | |
| C | 0.99 ± 3.6E-5 | 0.92 ± 3.66E-4 | 0.25 ± 0.0037 |
| S | 0.96 ± 0.00103 | 0.87 ± 6.69E-5 | 0.34 ± 0.00020 |
| 345 nm | | | |
| C | 0.9 8 ± 3.33E-5 | 0.92 ± 4.66E-4 | 0.24 ± 0.003 |
| S | 0.95 ± 1.35E-4 | 0.87 ± 8.56E-4 | 0.46 ± 8.25E-4* |
| 350 nm | | | |
| C | 0.98 ± 2E-4 | 0.93 ± 2.25E-4 | 0.22 ± 3.21E-4 |
| S | 0.83 ± 0.00113* | 0.86 ± 9.16E-5* | 0.47 ± 9.06E-4* |
| 355 nm | | | |
| C | 0.99 ± 0.0014 | 0.94 ± 7.21E-4 | 0.24 ± 0.0058 |
| S | 0.86 ± 0.0023* | 0.88 ± 6.41E-4* | 0.45 ± 8.25E-4* |
| 360 nm | | | |
| C | 0.92 ± 1.61E-4 | 0.94 ± 2.5E-5 | 0.36 ± 9.87E-4 |
| S | 0.79 ± 9.16E-5* | 0.86 ± 3.33E-5* | 0.48 ± 9.16E-5* |
| 365 nm | | | |
| C | 0.92 ± 0.0017 | 0.94 ± 9.16E-5 | 0.36 ± 0.0039 |
| S | 0.79 ± 5.65E-4* | 0.85 ± 2.25E-4* | 0.47 ± 2.79E-4* |

C: fluorescence intensity (a. u. – arbitrary unit) of control aorta intima;
S: fluorescence intensity (a. u. – arbitrary unit) of stressed aorta intima.
Fluorescence intensity values are expressed as mean ± SD2 for n = 8.
*P < 0.05
vs. control.

different rabbits subjected to hypodynamic stress. The recorded spectra are the sum of up to 8 individual spectra normalized to unit area after background subtraction and correction for the spectral response of the system. During measurements, the specimens were moistend with Ringer solution.

**Statistical analysis**

Statistical calculations were made using SPSS/w 12.0 (Statistical Package for Social Sciences, SPSS Inc., the USA) and Windows Excel Statistic packages.

Statistical comparison was performed using the Student's t test to analyse the differences in fluorescence intensities at selected excitation and emission wavelengths of each group of experimental samples (control and stressed aorta). The value of $P < 0.05$ was considered to be significant (Table).

## RESULTS

In the internal wall of the aorta of rabbits affected by hypodynamic stress we detected pyknosis and invagination of the nucleus of endothelial cells, thickening of the intima in some places and incorporation of cholesterol, a fragmented and disintegrated elastica interna, and disruption of the plasmalemma and the basement membrane. The subendothelial layer consisting of collagen and elastin fibers was disintegrated (Fig. 2A).

Characteristics of fluorescence spectra of the *ex vivo* control aorta intima and the aorta intima affected by hypodynamic stress at varying excitation wavelengths (340–410 nm) were recorded in the spectral range of 375–675 nm. Figure 3 shows fluorescence spectra of the control aorta intima and the aorta intima affected by hypodynamic stress at different excitation wavelengths. Experimental data were normalised to the maximum of the fluorescence intensity, and the variation in the shape of fluorescence spectra was analysed. Increasing the excitation wavelength, the fluorescence spectra were shifting to the red spectral range. For 340–360 nm excitation wavelengths, two typical fluorescence peaks near 445 and 490 nm were observed in the fluorescence spectra of both the control and stressed aorta intima, while in case of exciting fluorescence at longer wavelengths, only a single peak near 490 nm was characteristic. In the intima of aorta of control rabbits, the fluorescence band near 445 nm at shorter excitation wavelengths looked more expressive than the fluorescence band near 490 nm. On the other hand, in stress-affected aorta intima, the fluorescence band near 445 nm at shorter excitation wavelengths was less, and the fluorescence band near 490 nm was more pronounced than in the control intima of the aorta (Fig. 3). Noticeable slopes of the spectral curves were detected. Fluorescence of the stressed aorta showed a much broader fluorescence curve slope to the longer wavelength region, while fluorescence spectra of the control aorta at longer wavelengths showed a fast fall-off. Excited at longer wavelengths, the fluorescence band near 445 nm showed a fall-off and fluorescence near 490 nm a rise in both control and stressed aorta walls (Fig. 3). Using 340 and 345 nm excitation wavelengths, a slight variation of fluorescence spectrum shape at around 390 nm was observed for stress-affected aorta intima. Statistical analysis of the data showed that in the intima of stress-affected aorta, the intensity of fluorescence bands near 445 nm (350–365 nm excitation), 470 nm (350 nm, 360–365 nm excitation) was significantly ($P < 0.05$) lower, while fluorescence intensity at 570 nm

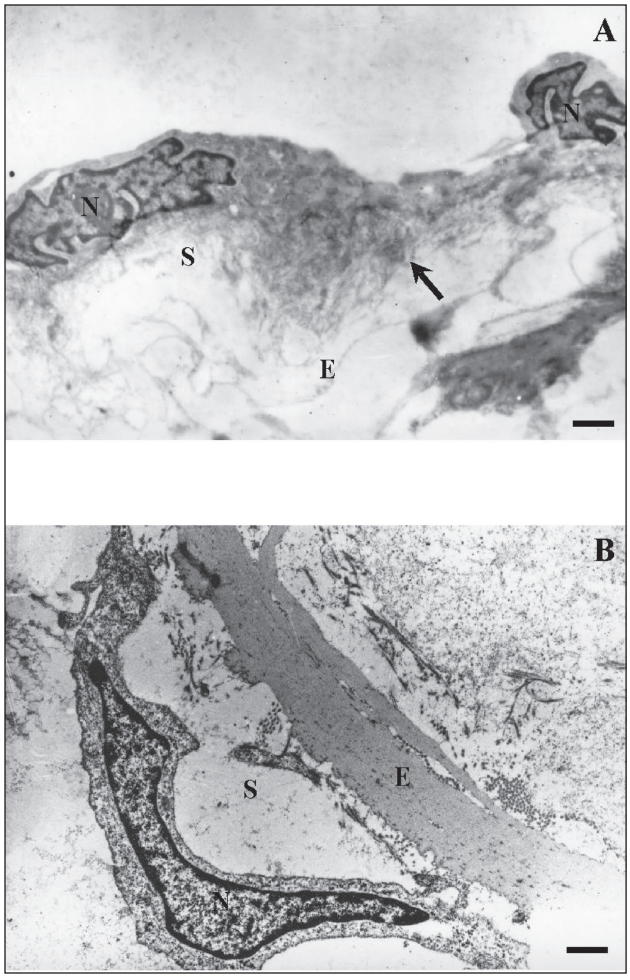


**Fig. 2.** Ultrastructure of thoracic aorta intima in rabbits affected by hypodynamic stress (A) and in control rabbits (B). A – pycnosis and invagination of the nucleus (N), intima tickening, cholesterol incorporation (the finger index shows cholesterol), subendothelial layer (S), elastica interna (E) are disintegrated. Scale bar = 10 μm

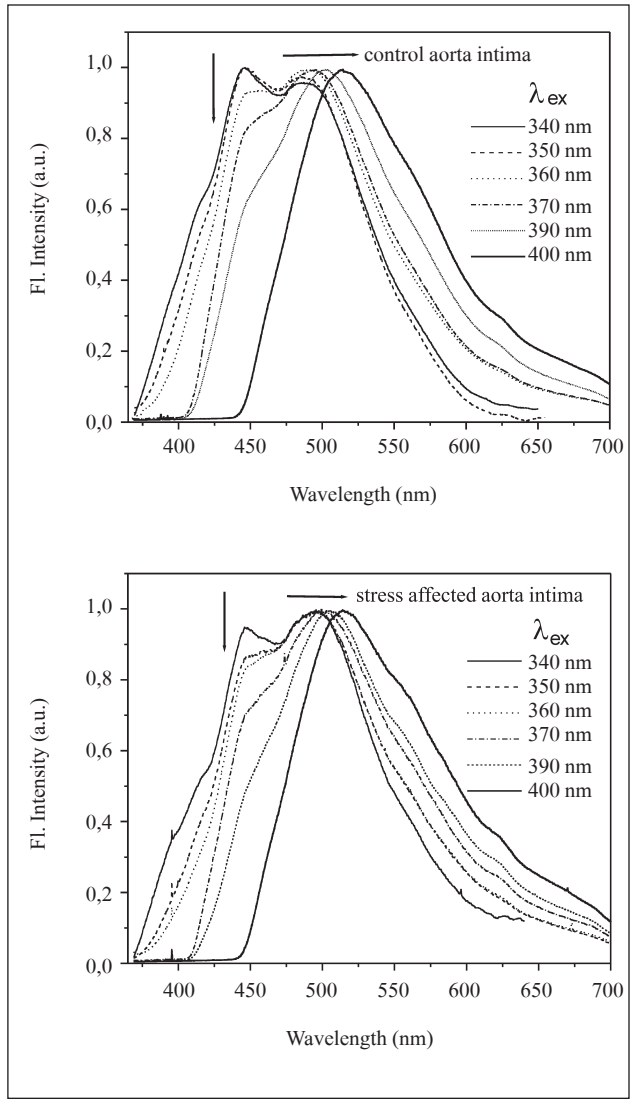


**Fig. 3.** Fluorescence spectra of control aorta intima and of aorta intima in rabbits affected by hypodynamic stress at different excitation wavelengths (λexc). All fluorescence spectra are normalized to their fluorescence intensity (a.u. – arbitrary unit) maximum. Vertical arrow indicates a decrease of fluorescence peak near 445 nm and horizontal arrow shows the shift of the total spectrum to a longer wavelength region with increasing excitation wavelength

(345–365 nm excitation) increased ($P < 0.05$) as compared with the intima of the control aorta (Fig. 4, Table). To determine the spectral regions presenting significant fingerprints for changes in the structure and content of tissue fluorphores, we calculated differential autofluorescence excitation-emission matrices (EEMs) (Fig. 5) by subtracting the EEM of an affected thoracic aorta from the EEM of a control thoracic aorta wall. The control aorta intima was characterised by a more intensive fluorescence at short wavelengths (around 445 nm), while stressed aorta intima showed a pronounced fluorescence for longer wavelengths (at around 570 nm) (Fig. 5).

## DISCUSSION

Optical spectroscopy has the potential of being selective as well as sensitive with respect to the detection of various molecular species and can be used for medical diagnostics. Laser-induced fluorescence spectroscopy in particular may prove useful in early detection of diseased tissue [10]. Thus, the aim of our investigation was to evaluate the possibilities of fluorescence spectroscopy for the recognition of early structural changes of the intima in the thoracic aorta of experimental animals subjected to hypodynamics. Microscopy data show that hypodynamics caused ultrastructural alterations in the aortic wall, associated with variations of endogenous fluorophores such as accumulation of lipids and the disintegration of the elastica interna and the subendothelial layer consisting of collagen and elastin (Fig. 2A). According to literature data, different tissue alterations or pathological conditions may also be associated with variations in biological fluorophores (collagen, elastin, NADH, lipids, etc.) within separate layers, or attended by the domination of a concrete fluorophore which often appears to be specific to the abnormal tissue [10]. Thus, tissue fluorescence may reflect changes in the tissue metabolic process, associated with the disturbance of the biochemical composition of the tissue, such as endogenous fluorophores. The spectral shape of fluorescence is often sufficient to distinguish between normal and diseased tissue in different bodyline systems like brain [11], gynaecological tract [12], vascular site [13] and others [14]. Our experimental study

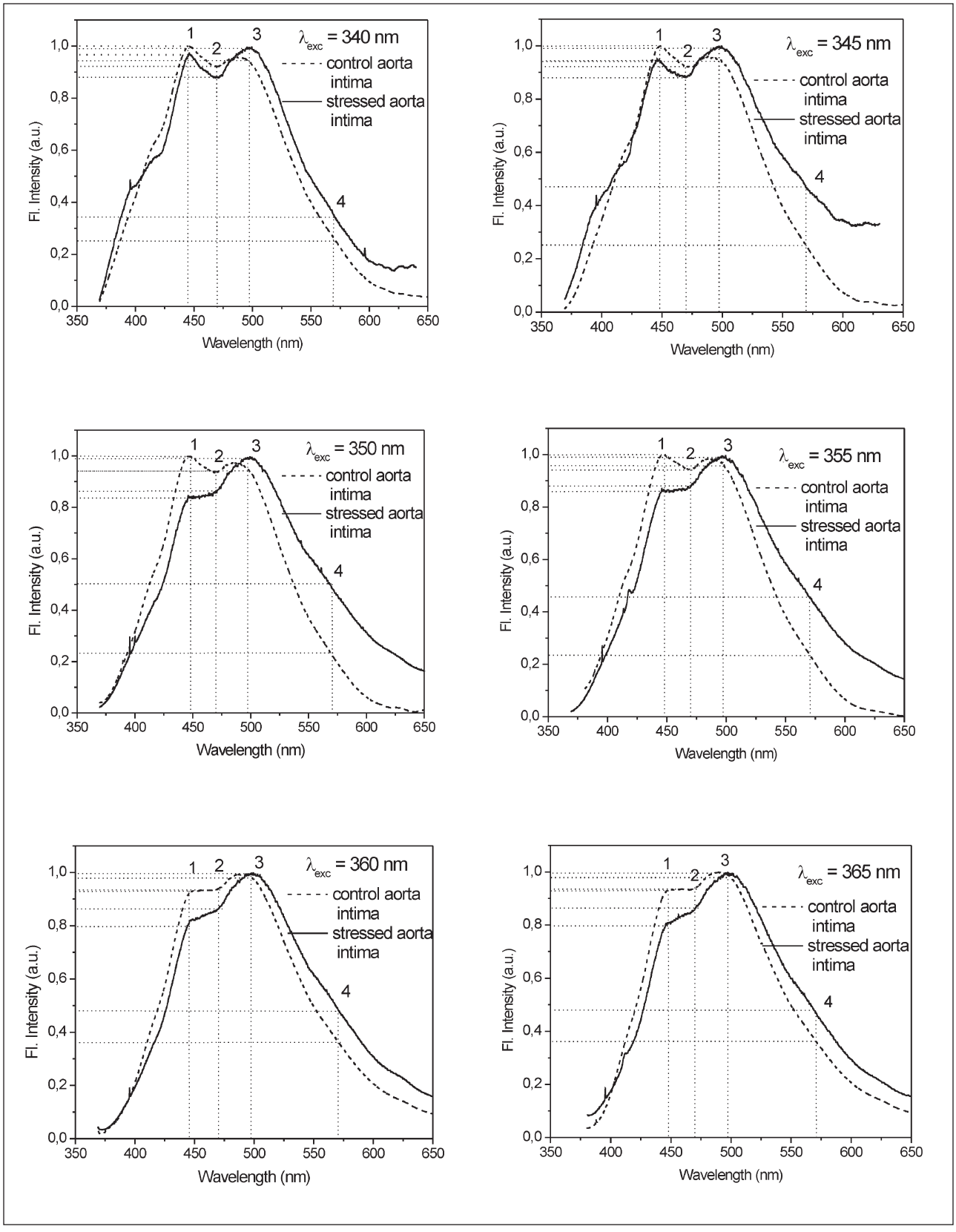


**Fig. 4.** Mean intensity of fluorescence spectra of hypodynamic stress-affected aorta intima (n = 8) and control aorta intima (n = 8) under different excitation wavelengths (λexc). Dotted grids show the main variations in fluorescence spectral shape at around 445 (1), 470 (2), 490 (3), and 570 (4) nm. The mean values of statistically significant ($P < 0.05$) fluorescence intensities (a.u. – arbitrary unit) as compared with the inner wall of control and stressed aorta are given in Table

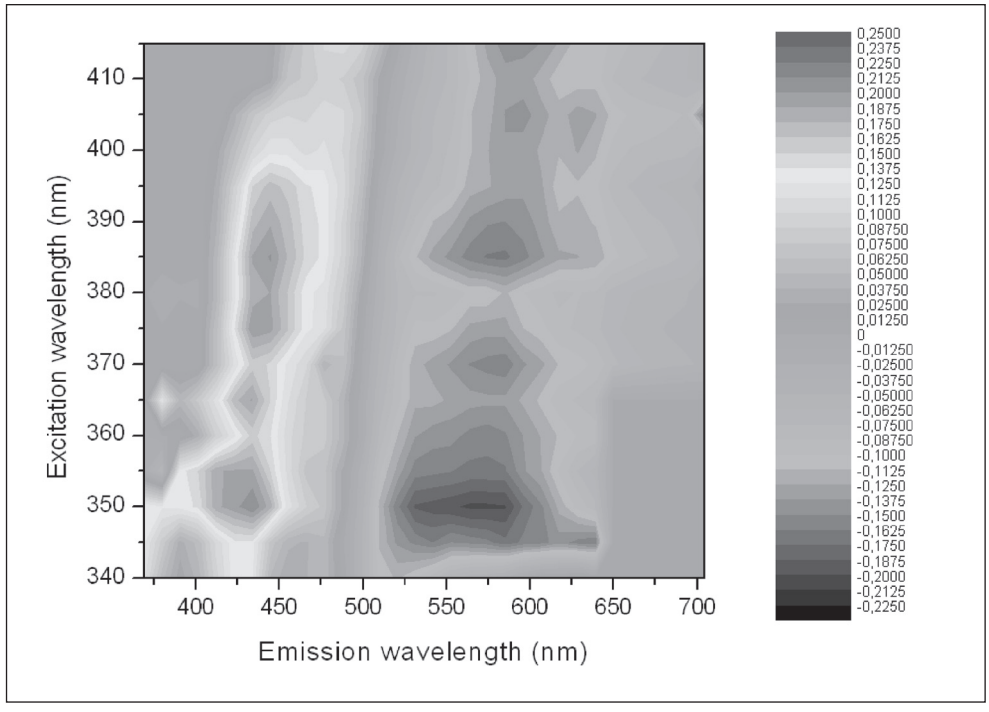


**Fig. 5.** Differential 2-dimensional excitation-emission matrices (EEMs). The control aorta intima shows a more intense fluorescence at short wavelengths (445 nm). Stressed aorta intima shows expressive fluorescence at longer wavelengths (570 nm)

demonstrated that changes in the spectral line shapes of fluorescence are related to alterations in the endogenous fluorophores of the inner side of the artery wall, caused by hypodynamic stress. We found that for 340–360 nm excitation wavelengths, the fluorescence band near 490 nm in the wall of the stress-affected aorta was more pronounced than the band near 445 nm, in comparison with that in the intima of the control aorta (Fig. 3). The use of different excitation wavelengths allowed for a selective excitation of various tissue fluorophores and thus for the delineation of most relevant fluorescence features that characterise the structural state of the tissue. The ability to select an optimal excitation wavelength is of great importance as it shows which tissue fluorophore mostly contributes to tissue fluorescence, affecting its shape and intensity. Different (or several) excitation wavelengths are used to activate tissue fluorescence, and then the recorded spectra are classified according to histological findings [15]. The recorded emission signals for different excitation wavelengths can be plotted as excitation–emission matrices that characterise the properties of tissue fluorescence as a multifluorophore system [16, 17].

The differential excitation-emission matrix constructed by us allowed to identify two main discriminative regions where differences between the fluorescence of control and stress-affected aorta intima occurred (Fig. 5). The control aorta intima showed a pronounced fluorescence at around 445 nm, while an increase in fluorescence at around 570 nm corresponded to the aorta intima affected by hypodynamic stress. On the basis of ultrastructural results (Fig. 2A, B) and according to literature data [5, 18], the fluorescence peak near 445 nm, fluorescence intensity at around 470 nm, and the peak near 570 nm may be related to protein elastin, NADH and lipopigments, respectively (Fig. 4, Table). Thus, the disintegrated elastica interna and the subendothelial layer consisting of collagen and elastin may by associated with a decreased concentration of elastin as endogenous fluorophore and cause a statistically significant ($P < 0.05$) lowered intensity of fluorescence emission at around 445 nm (excitation 350–365 nm) for the stress-affected aorta intima (Table). Banga et al. [19] supported the hypothesis that the fluorescent substances that accumulate in the human atherosclerotic aorta are closely related to the intimal plaque and take the form of "atherofluorescent" component pigments which are directly related to the degeneration of elastin. The maximum activation / emission peaks of these pigments were determined near 350 / 405 nm and 380 / 450 nm, respectively. The substance contributing to fluorescence in the near-UV region from an artery wall has been identified by Baraga et al. [20] as NADH (470 nm emission). According to Wagnieres et al. (18), the contribution to the tissue fluorescence from NADH is marked (near 470 nm emission) by excitation at about 350 nm wavelength. Thus, a significantly ($P < 0.05$) lower fluorescence intensity near 470 nm (350 nm, 360–365 nm excitation) of the stressed aorta intima as compared to the control aorta may be associated with a decreased concentration of NADH. This endogenous fluorophore as a major bioenergy substratum is also important in maintaining the normal ultrastructure of cells in tissues. The above-mentioned impairments in the ultrastructure of the stress-affected aorta intima support the hypothesis that a lower fluorescence intensity near 470 nm may be associated with a decreased amount of NADH. The statistically higher ($P < 0.05$) (Table) intensity of fluorescence of the stress-affected aorta intima (Fig. 4) in comparison with that of control aorta at around 570 nm (excitation 345–355 nm) is likely related to the fluorescence of lipids. This supports our data showing the incorporation of lipids in

endothelial cells (Fig. 2A) and findings of others authors [18] who from excitation of the artery wall at around 345–355 nm determined the maximum fluorescence intensity near 570 nm. Filippids et al. [21] also suggest that during the early stage of atherosclerotic lesions, the fluorescence of the tissue is mainly influenced by lipids. Advanced human atherosclerotic lesions as seen in unselected autopsy specimens often contain surprisingly little lipids and much collagens [22]. The strong fluorescence of collagen at 390 nm (emission) is related to an advanced atherosclerotic process [23, 24]. In our experiment, fluorescence intensity was weak (Fig. 4) at 390 by 340–345 nm wavelength of excitation.

## CONCLUSIONS

Incorporation of cholesterol as an endogenous fluorophore in stressed aorta intima and an increase in the intensity of fluorescence at 570 nm wavelength were detected in comparison with the control aorta. A statistically significant ($P < 0.05$) decrease in fluorescence intensity at 445 nm in stress-affected thoracic aorta intima compared to the control aorta was accompanying the fragmented and disintegrated subendothelial layer and elastica interna. Data of fluorescence EEMS also showed an expressive fluorescence of stressed aorta intima at around 570 nm, but an intensive fluorescence was shown by the control aorta intima at short wavelengths (around 445 nm). All these facts suggest that the above-mentioned ultrastructural damages in the intima of the thoracic aorta of stressed rabbits are possible to recognise from variations of fluorescence intensity under laser-induced fluorescence spectroscopy.

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**Marija Kušleikaitė, Roaldas Gadonas, Dalia Kaškelytė, Sigitas Stonkus, Rimantas Benetis, Saulius Kušleika, Vitalijus Karabanovas**

## TRIUŠIŲ KRŪTINĖS AORTOS INTIMOS PAŽEIDIMŲ (*EX VIVO*), SUKELTŲ HIPODINAMIJOS, ATPAŽINIMO GALIMYBĖS LAZERINĖS SPEKTROSKOPIJOS METODU

*S a n t r a u k a*

Fluorescencinės spektroskopijos metodu buvo tirti triušių krūtinės aortos intimos pažeidimai, atsiradę dėl hipodinaminio streso. Tam tikslui Fiodorovo (1991) metodu buvo sukelta 48 parų hipodinamija šinšilos veislės triušiams, juos imobilizuojant metaliniuose labai ankštuose narveliuose. Krūtinės aortų intimų ultrastruktūros (8 bandomųjų ir 8 kontrolinių triušių) tirtos elektroniniu mikroskopu (Philips-300), o jose esančių fluoroforų charakteristikos – lazerinės spektroskopijos metodu panaudojant Nd lazerį. Visų aortų fluorescencinių spektrų charakteristikos (žadinant 340–415 nm bangomis) buvo tirtos 375–675 nm spektriniame ruože. Labiausiai reikšmingas ($p < 0,05$) hipodinamijos pažeistų aortų (lyginant su sveikomis) fluorescencijos intensyvumo padidėjimas (žadinant 345–365 nm bangomis) rastas ties 570 nm banga, o sumažėjimas – ties 445 nm (žadinant 350–365 nm bangomis). Šie skirtumai išryškėjo ir žadinimo-emisijos matricose: fluorescencijos intensyvumo padidėjimas ties 445 nm spektro banga sveikose aortose, o ties 570 nm – streso pažeistose. Kartu su fluorescencijos intensyvumo kaita streso paveiktose aortose pastebėtas intimų sustorėjimas, cholesterolio (kaip aterosklerozinio pažeidimo komponento) sankaupos, poendotelinio sluoksnio, vidinės elastinės membranos irimas. Pateikti tyrimo duomenys rodo, jog yra galimybė hipodinamijos sukeltus krūtinės aortos ultrastruktūros pažeidimus atpažinti lazerinės mikroskopijos metodu.