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MORPHOMETRIC ANALYSIS OF MYOCARDIAL AND INTERSTITIAL CONNECTIVE TISSUE IN THE HEROIN ADDICTS: A CASE-CONTROL STUDY

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Sudden deaths are mostly caused by substance abuse and overdose, whether they were used separately or combined with other substances having depressor effect on the central nervous system. Apart from the pathology associated with the central nervous system and the lungs, a large portion in sudden death occurrence in opiate abusers lies also in the pathology of cardiovascular system, especially the heart. A group of 42 long-term heroin addicts was observed (35 men and 7 women), aged 18-48 years, whose sudden death was related to heroin abuse, whether heroin taken intravenously (38 cases) or by sniffing (4 case). Myocardial tissue samples were processed with the modified Movat's staining procedure and analysed statistically. In the current study, standard histological examinations of the heart muscle in heroin addicts found cardiomyocyte hypertrophy and interstitial and/or perivascular fibrosis with statistical significance ($p < 0.001$) in comparison with the control group. In 24% (10 cases) of all examined cases, histological picture in the heart muscle matching the picture of the acquired cardiomyopathy was determined. We believe that in cases of repeated intoxication, even in the doses of heroin that are not inherently lethal, sudden changes in hemodynamics and disturbances in the rhythm of operation of such altered and more vulnerable heart muscle can affect the occurrence of sudden cause of death.

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Key words: *drug abuse, heroin, myocardial fibrosis, cardiomyocyte hypertrophy*

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Introduction

Substance abuse is a major social and health issue predominantly affecting younger population (1-4). Sudden deaths are mostly caused by substance abuse and overdose (5-7), whether they were used separately or combined with other substances having depressor effect on the central nervous system.

According to numerous studies performed on autopsy material, it is clear that besides the pathology associated with the central nervous system and the lungs, a large portion in sudden death occurrence in opiate abusers lies also in the pathology of cardiovascular system, especially the heart.

Numerous studies are dealing with this issue, and especially those related to heart and heart muscle alterations caused by cocaine and amphetamines abuse (8-14). These substances, acting as cardiovascular system stimulants, lead to the increase in heart rate and vasoconstriction of the coronary blood vessels, which in susceptible individuals may lead to disturbances in cardiac rhythm and/or myocardial infarction (4). In addition, it was noticed in the autopsy material that in such cases there is a slight chronic inflammatory heart muscle infiltration, multiplication of connective tissue (6), as well as cardiomyocyte hypertrophy (15-17).

The issue of cardiac pathology in relation to heroin abuse has been insufficiently considered, despite the fact that at the present time heroin addiction is very important and growing problem, especially among young people. Myocardial ischemia and

infarction as well as a poorly characterized cardiomyopathy have been reported (18, 19).

The aim of this paper was to present the morphological changes in the heart muscle and their relationship with the immediate cause of death in heroin addicts.

Materials and methods

A group of 42 long-term heroin addicts was observed (35 men; 7 women), aged 18-48 years, whose sudden death was related to heroin abuse, whether heroin taken intravenously (38 cases) or by sniffing (4 cases). For 11 cases there are no data about the length of the heroin usage, 13 cases used heroin < 5 years, 11 cases used heroin for 5-10 years, and 7 cases used heroin > 10 years.

Autopsy results of 10 heroin addicts (24%) showed hypertension or hypertrophy of left cardiac ventricular wall, and these cases were not further examined.

The control group included 10 persons, aged 22–35 years, for whom the data on antemortem opiate abuse and existence of cardiac disease was not obtained and the cause of their death was the traffic trauma (severe traumatic shock, haemorrhage or polytrauma).

All autopsies were performed within 48 hours of the death.

Histological examination

Cardiac muscle samples were systematically sampled from standard positions (front, rear and side wall of the left ventricle, cranial part of the interventricular septum, anterior and posterior wall of the right ventricle). From each standard position, three cardiac muscle tissue samples were taken. The tissue samples were stored in a neutral, buffered 4% formalin solution, for 18-24 hours, dehydrated in ethanol of progressive concentration and embedded in paraffin wax. From the paraffin blocks, the tissue was cut into 4-5μm thin samples, routinely stained with the hematoxylin and eosin method, while the cardiac muscle fragments were processed with the modified Movat's staining procedure also. The significance of the modified Movat's staining usage is the easier and shorter procedure lasting (about 70 min); and shows minimal overstainig and maximal efficiency in differential histochemical staining (20). Microscopic analysis was performed by Leica DM1000 (Wetzlar, Germany), with digital microscopic camera Leica EC3 (Wetzlar, Germany) with Leica LAS EZ imaging software V1.8.0.

Morphometric and image analysis

The thickness of cardiomyocytes was considered, as well as the ratio between the areal fractions of the cardiomyocytes and fibrous connective tissue. The morphometric analysis was performed in ImageJ program ver 1.50

http://rsb.info.nih.gov/ij/index.html).

Before analysis, system was calibrated first. Digital cross-sectional views of modified Movat's pentatachrome stain were observed and photographed under magnification x200 and in a further procedure used to measure the stereological parameters of connective tissue in myocardium. By random selection method, 30 fields of vision were selected per one case. Across digital images of the aforementioned field of view is superposed is a digital line network system generated by the software using the options Plugins-Analyse-Grid (Figure 1).

The volume density of the connective tissue (VVT) was calculated as the number of points affecting connective tissue (PVT) in the appropriated field of vision and the total number of points of the test system (PT) (VVT = PVT / PT). From the obtained values of the volume density of all analyzed fields of vision, the average value of the volume density of each case was expressed in percentages, and mean values per case were shown.

Morphometric analysis of the cardiomyocytes was performed under magnification x400. The thickness of 10 cardiomyocytes randomly selected per field was measured and mean values per case were shown (Figure 2).

Statistical analysis

Differences among groups were examined by F–Test for testing statistical significance of the variance, and by Student's t–test for two independent samples. Values are expressed as the mean with standard error (SE). Values of $p \leq 0.05$ were considered statistically significant. All statistical analyses were performed with software Jandel SigmaStat 2.0.

Results

Myocardial Hypertrophy

F–Test for testing statistical significance of the variance of two independent samples revealed the existence of statistically significant difference in the study groups ($p < 0.05$). Consequently, testing the difference between the average values of cardiomyocytes thickness in the group of addicts and the control group was performed using the Student's t–test for two independent samples with unequal variances. The results of this test showed that the average thickness of cardiomyocytes in the group of heroin addicts was statistically and significantly higher compared to the control group ($p = 0.001$; Table 1) which represents the moderate intensity difference (Cohen's $d = 0.23$).

Ratio between the areal fraction of the interstitial connective tissue and areal fraction of the myocardium

F–test for testing statistical significance of the variance of two independent samples also indicated the existence of statistically significant differences in the groups studied ($p < 0.001$). Student's t–test for two independent samples with unequal variances showed that the average areal fraction of interstitial connective tissue in relation to the areal fraction of

cardiomyocyte, in the group of heroin addicts was statistically significant higher than in the control group ($p < 0.001$; Table 1) which represents the moderate intensity difference (Cohen's $d = 0.4$).

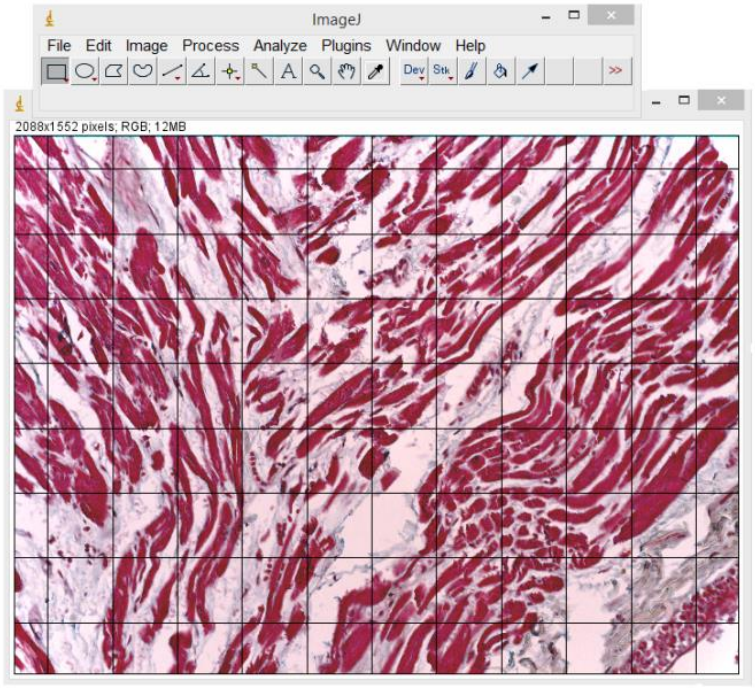

Figure 1. Measuring of the stereological parameters of connective tissue in myocardium (modified Movat's; original magnification x400) in ImageJ program

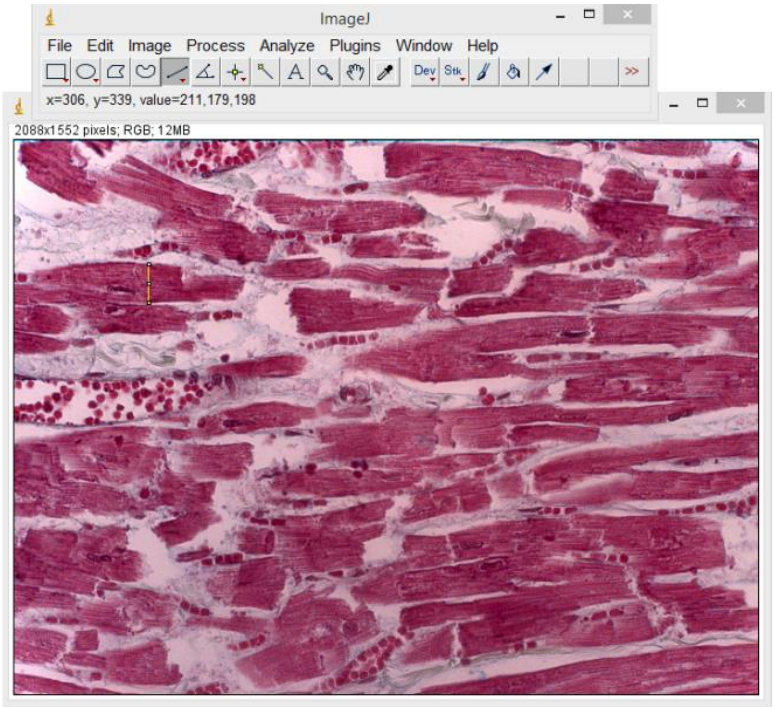

Figure 2. Morphometric analysis of the cardiomyocytes (modified Movat's; original magnification x200) in ImageJ program

Table 1. Myocardial thickness and ratio between the areal fraction of the interstitial connective tissue and areal fraction of the myocardium of the evaluated cases

№	Parameter			
	Myocardial thickness (μm)		Interstitial connective tissue/ myocardium ratio (%)	
	Heroin addicts	Control group	Heroin addicts	Control group
1.	14.414	13.686	41.45	33.88
2.	14.814	12.018	33.63	32.34
3.	14.265	12.180	35.38	34.79
4.	15.789	11.234	40.40	30.42
5.	16.994	12.142	39.03	30.85
6.	16.298	10.828	38.18	31.73
7.	14.245	11.985	44.29	29.79
8.	15.452	10.816	43.63	30.01
9.	15.958	11.169	40.30	29.15
10.	15.819	10.340	36.18	29.00
11.	12.644		40.00	
12.	8.022		38.16	
13.	7.588		29.52	
14.	10.774		31.66	
15.	14.376		49.48	
16.	12.592		40.53	
17.	7.936		40.09	
18.	9.209		44.60	
19.	7.298		45.06	
20.	11.006		40.68	
21.	15.144		45.81	
22.	16.488		44.44	
23.	15.06		30.63	
24.	15.725		32.82	
25.	15.421		33.53	
26.	14.439		35.67	
27.	15.652		31.75	
28.	13.547		34.06	
29.	15.323		31.07	
30.	14.731		35.43	
31.	14.389		33.13	
32.	17.06		31.59	
Mean	13.7*	11.6	34.9*	33.4
SE	0.51	0.31	0.7	0.6

SE – Standard error
* – heroin addicts vs. control group p = 0.001

Histological examination of other organs, in most cases, among other findings, found acute blood stasis in the internal organs and brain swelling. Hemorrhagic pulmonary edema was found in 40%

of the tested cases, while in others cases, pulmonary edema was found in low to medium degree of severity, without hemorrhagic component. In all cases in the group of heroin addicts the interstitial fibrosis was found (Figure 3), while in 22 cases (70%), in addition to the interstitial, the perivascular fibrosis was also found (Figure 4).

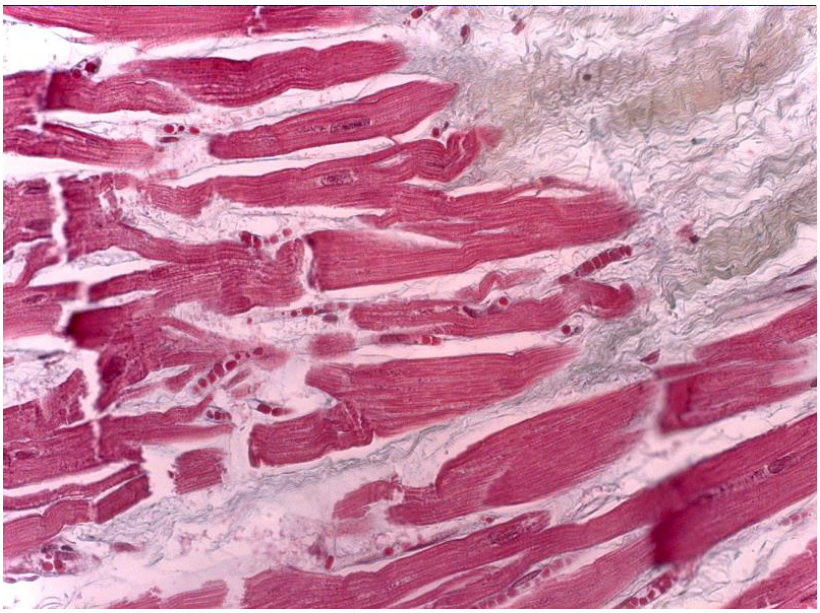

Figure 3. Fibrosis in the interstitium (modified Movat's; original magnification x400)

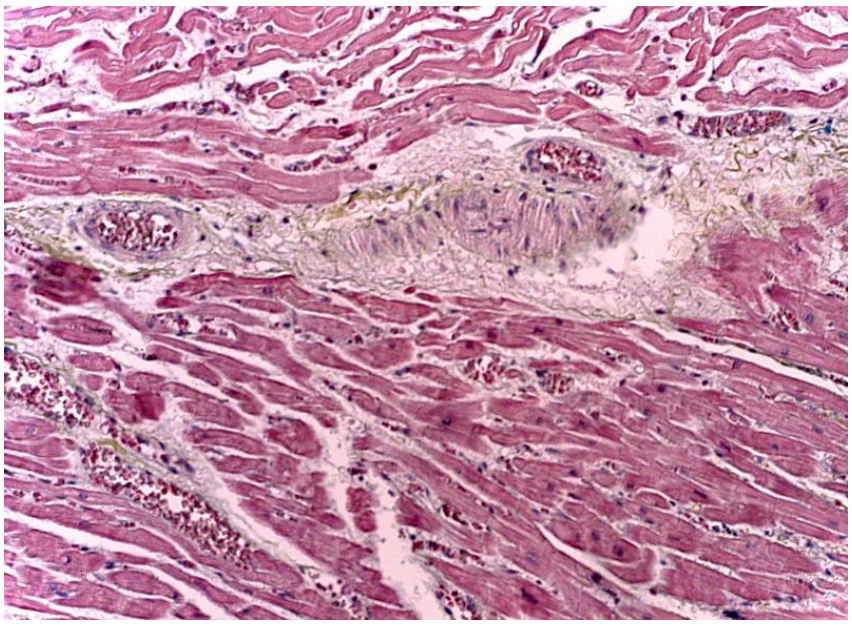

Figure 4. Concentric fibrosis around small artery (modified Movat's; original magnification x200)

Six cases (19%) from the group of heroin addicts also had lipomatosis, which was predominantly expressed in the region of the front wall muscle of the right ventricle. There was also an increase in the number of mast cells in the interstitium of the heart muscle particularly in the level of the

multiiplied connective tissue surrounding the small blood vessels in heroin addicts compared to the control group.

In the examined group of heroin addicts, the concentration of morphine measured in femoral blood sample ranged from 0.067 to 2.03mg/L, and 6–monoacetylmorphine (6–MAM) concentration ranged from 0.06 to 0.87 mg/L. In 10 cases (31.25%), ethanol was detected in blood sample in the range from 0.09‰ to 3.05‰, while diazepam in therapeutic or lower concentration together with heroin metabolites were detected in 9 cases (28.13%).

Discussion

Looking at data from the literature, it can be observed that in explaining the occurrence of death among heroin addicts the focus is on the malignant hemorrhagic pulmonary edema as a consequence of acute heroin intoxication. Although it is unknown precisely why it arises, the possible reasons for occurrence of the malignant hemorrhagic pulmonary edema are considered to be hypoxia caused by the increased capillary permeability, cardiomyocyte contractility depression, centrally induced respiratory depression, primary toxic heroin effects on the alveolar-capillary membrane and acute anaphylactic shock (21). The toxic effects of heroin and its direct metabolites directly to the heart muscle with an aim to explain cardiac failures as the possible cause of death in heroin addicts were considered sporadically (1-5). The tests conducted so far were mainly based on existing chronic inflammatory changes in the heart muscle, while morphological changes resulting from the inflammatory processes, in explaining the immediate cause of death in heroin addicts, were generally perceived as supportive rather than dominant (15, 22).

In the current study, standard histological examinations of the heart muscle in all cases so far found cardiomyocyte hypertrophy and interstitial and/or perivascular fibrosis, while in 24% of the examined cases, histological picture in the heart muscle matching the picture of the acquired cardiomyopathy was determined (18-19). Significance of the difference between the areal fractions of the connective tissue in the tested group of heroin addicts and the control group was established, as well as statistically and significantly thicker cardiomyocytes in the group of heroin addicts ($p < 0.001$), which is in agreement with the literature data and the pathohistological mechanism of the cardiac muscle response to toxic effects of heroin. Namely, the morphological changes in the cardiac muscle that occur in response to the effects of heroin are probably of hypoxic type and are based on sporadic death of certain cardiomyocytes along with the hypertrophy of the preserved cardiomyocytes and reduction in their total number. In the process of remodeling the damaged myocardium after the death of cardiomyocytes, it is in these places that fibrosis occurs in the process of excessive accumulation of extracellular matrix (ECM), predominantly composed of collagen.

Research conducted on experimental animals determined that heroin and morphine reduce blood pressure and heart rate, explained by histamine release, especially from mast cells (15, 23). In 60% of studied sudden death cases among heroin addicts, the routine processing of the tissue did not establish the existence of malignant hemorrhagic pulmonary edema, the occurrence of which require a certain period of time. Also, the fact that the finding of the increased number of mast cells in the cardiac muscle, where the impact of histamine from their depos in the case of the repeated intoxication can further contribute to the stated cardiovascular abnormalities (15).

Conclusion

Test results clearly indicate that the effect of chronic heroin abuse on the cardiac muscle causes cardiomyocyte hypertrophy and multiplication of fibrous tissue. In cases of repeated intoxication, especially in patients in the methadone therapy, even in the doses of heroin that are not inherently lethal, sudden changes in hemodynamics and disturbances in the rhythm of operation of such altered and more vulnerable heart muscle, can affect the occurrence of sudden cause of death. The disruption in morphologically altered cardiac muscle functioning in repeated heroin intoxication cannot be a priori taken only as a supportive factor for the occurrence of death, but as a competitive cause of death.

Namely, in most cases, and especially in cases where heroin was taken together with alcohol and/or benzodiazepines, morphological changes in the heart muscle can be considered as a significant factor in the occurrence of death.

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Prikaz bolesnika

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MORFOMETRIJSKA ANALIZA MIOKARDIJALNOG I INTERSTICIJALNOG VEZIVNOG TKIVA HEROINSKIH ZAVISNIKA: STUDIJA SLUČAJEVA

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Naprasne smrti najčešće su izazvane zloupotrebom raznih opioidnih supstanci i predoziranjem, bez obzira da li su korišćene ponaosob ili u kombinaciji sa drugim supstancama, izazivajući depresorni efekat na centralni nervni sistem. Pored patologije centralnog nervnog sistema i pluća, veliki broj slučajeva naprasnih smrti opioidnih zavisnika leži u patologiji kardiovaskularnog sistema, naročito u patologiji srca. Analizirana je grupa od 42 dugogodišnja heroinska zavisnika (35 muškaraca i 7 žena), starosti od 18 godina do 48 godina, čije su iznenadne smrti bile u vezi sa zloupotrebom heroina, bez obzira da li je heroin uzet intravenski (38 slučajeva) ili ušmrkavanjem (4 slučaja). Isečci tkiva miokarda obrađivani su modifikovanom Movatovom procedurom bojenja i statistički analizirani. U aktuelnoj studiji, standardna histološka analiza srčanog mišića heroinskih zavisnika pokazala je zadebljanje kardiomiocita i umnožavanje intersticijale i/ili perivaskularne fibroze sa statistički signifikantnom razlikom ($p < 0,001$) u poređenju sa kontrolnom grupom. U 24% (10 slučajeva) svih ispitivanih slučajeva histološka slika srčanog mišića odgovara stečenoj kardiomiopatiji. Smatramo da u slučajevima ponavljanih intoksikacija, čak i u slučajevima gde aplikovane doze heroina nisu bezuslovno letalne, nagle promene u hemodinamici i distribuciji u ritmu rada izmenjenog i vulnerabilnog srčanog mišića mogu uticati na nastanak naprasne smrti.

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Ključne reči: *zloupotreba droge, heroin, miokardijalna fibroza, hipertrofija miokardiocita*