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CHANGES IN THE MITOCHONDRIAL APPARATUS OF CARDIOMYOCYTES UNDER THE INFLUENCE OF OPIOID IN THE EXPERIMENT[6]

Abstract:
This scientific work presents a theoretical summary and a new solution of the scientific task to establish peculiarities of the structure and some biochemical parameters of blood and organs of white rat's myocardium under normal conditions and under the prolonged effect of an opioid. The experiment was carried out on 53 male white rats 130-210 gr. body weight. The animals were divided into the 3 groups – experimental group (30 animals), control group (18

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animals) and 5 animals to study the norms before the experiment. Application of the optical and electron microscopy confirmed, that the white rat's myocardium is formed by muscle fibers that branch out and intertwine between themselves thus forming a peculiar reticulum. Myofibrils in their turn consist of a chain of sarcomeres restricted by Z-lines, each containing 2-3 mitochondria of all the 3 types. The first signs of impairment of the myocardium microstructure are noticeable after 7 days of nalbuphine injections to the white rats. During the following 35 days of the experiment pathological changes increase and are manifested through impairment of the well-defined structure of the myocardium, fragmentation of the muscle fibers, evident perivascular swelling, diffuse sclerosis; the small caliber vessels are characterized by the evident hyperemia with the "sludge syndrome" observed in the lumens of microvessels.

Keywords:
myocardium, microvasculature, white rat, mitochondria, nalbuphine.

Introduction

Nalbuphine hydrochloride is a nonscheduled potential analgesic, which belongs to the group of agonist-antagonist opioid receptors, widely used in different branches of medicine (1). The increasing popularity of nalbuphine in our country is due not only to its efficiency as a pain-killer and accessibility as a nonscheduled drug but because of using with non-medical purpose too (2). The data about an abuse potential of nalbuphine and social and medical problems, related to these questions, were frequently raised up in the literature. Drug addiction has been widely spreading in Ukraine in the last decade. According to the WHO there were about 400 000 injection drug users in Ukraine. Each year the number of drug users in Ukraine increases by 8-9 %. The negative is the fact that 70-75% of the drug addicts are young people under 25 years of age (3-5). The group of opioid analgesics is widely used these days in cases of chronic pain syndrome. Narcotic analgesics are traditionally prescribed and produce a positive effect on the pain syndrome of the wounded in the battlefield. The wide use of narcotic substances, their growing distribution and the spread of drug addiction necessitates a detailed study of the effect of opioids on the human organism. However, most of the findings are based on the clinical use of the drugs without a sufficient experimental study, especially regarding the morphological changes, which is totally unacceptable. Taking into consideration professional literature one can come to conclusion, that there is a whole range of unresolved problems concerning the morphological restructuring of the myocardium under the effect of the opioid.

Still insufficiently studied remain the issues of macro-, micro- and ultrastructural peculiarities of the myocardium microvasculature. This is why the study of morphological changes in the myocardium and its microvasculature under the effect of opioids in the experiment remains to be exceptionally topical. On another hand, numerous data demonstrated different side effects, caused by a long-term nalbuphine administration, including morphological changes of different organs (6-10). Heart, brain, liver, and kidney, are first organs, aimed by opioids in a case of a long-term administration (11-13). In particular, well-expressed changes in rats heart microstructure under the routine light microscopy, as well as changes of biochemical parameters were revealed in our previous studies (14, 15). With the purpose of better understanding the key mechanism of these changes, the transmission electron microscopy was planned as the next stage of our project. The disturbance of microcirculation and cardiomyocytes dystrophy were market previously as are the key moments in heart pathology as consequences of nalbuphine administration. But pathophysiology of these changes remains uncertain. Thus, the current study aimed to evaluate the long-term effect of nalbuphine hydrochloride on rats heart ultrastructure, in particular, to obtain a new data about heart, vessels endotheliocytes and cardiomyocytes changes, using electron microscopy and scanning electron microscopy.

Aim of the study

Aim of the study: to establish histological and ultramicroscopic peculiarities of the structure and microvasculature of the myocardium of the white rat in physiologically normal state; to determine morphological changes in the microvasculature and structural organization of the white rat's myocardium under the effect of an opioid on the microscopic level; investigate morphological changes in the microvasculature and structure of the white rat's myocardium under the effect of an opioid on the ultrastructural level; carry out stereological analysis of the changes in angioarchitecture and quantitative parameters of the white rat's cardiomyocytes under the effect of an opioid on the micro- and ultrastructural levels

Material and methods

The experiment was carried out on 53 male white rats 130-210 gr. body weight. The animals were divided into the 3 groups – experimental group (30 animals), control group (18 animals) and 5 animals to study the norms before the experiment. The experiment was conducted in accordance with the provision of the European Convention for the protection of the vertebrate animals used for the experimental and another scientific purpose from

24.11.1986 and the approved by Ethical Committee or Institutional Animal Care and Use Committee Approval, protocol №3 from 20.03.2015. The animals of the experimental group were daily injected by nalbuphine hydrochloride according to scheme what were proposed by Onysko and co-authors (16) with weekly dose increasing from 8 mg/kg body weight to 35 mg/kg body weight. Control group animals were injected daily by 0,5 ml saline. Heart samples (less than 1 mm in each direction) were taken at the end of every week after the intraperitoneal injection of sodium thiopental (25 mg per 1 kg body weight). After immediate fixation in 2,5 % buffered glutaraldehyde, the samples were prepared for the electron microscopy following standard procedure (17,18). For studying and photographing of the samples a microscope UEMV – 100K was used at an accelerating voltage of 75 kV and magnification range x2000-x15000.

Volume fraction (volume density) of mitochondria was calculated as $Vv(m) = V(m)/V(c)$, were $V(m)$ – volume of mitochondria, $V(c)$ – volume of cardiomyocyte cytoplasm. The volume of mitochondria and volume of cardiomyocyte cytoplasm were estimated using Stepanizer stereology tool v.1.0 with points test system.

For the analysis of the sample distribution, the histogram analysis, indicators of excess and asymmetry, as well as q-q plot analysis were performed. Since the data we obtained were differed from the normal distribution, the results were presented as Me (25%, 75%), where Me is a median, 25% - 25th percentile, 75% - 75th percentile, in addition to the general table indicates the interquartile range (IQR) of the obtained data. The Mann-Whitney U-test was used to check the significance of the difference between the control and experimental animal groups. The level of significance was set at $p < 0.05$

LibreOffice Calc v.5.2.2.2 spreadsheets were used for initial analysis and drawing of data in the form of tables and graphs. Microsoft Office Excel 2007, the software InVivoStat ver.3.0 and SofaStat v.1.4.6 were used for statistical analysis.

Results

By application of the optical and electron microscopy confirmed, that the white rat's myocardium is formed by muscle fibers that branch out and intertwine between themselves thus forming a peculiar reticulum. The myocardium muscle fibers formed by mono- or binuclear cardiomyocytes that in their cross-section have a rectangular form are contractile cardiomyocytes, formed by the bundles of located in parallel myofibrils restricted by sarcolemma. Myofibrils in their turn consist of a chain of sarcomeres restricted by Z-lines, each containing 2-3 mitochondria. Cardiomyocytes are

interconnected with the aid of the intercalated disks. Fissures between cardiomyocytes are filled with the loose connective tissue with the nerves and microvasculature components presented by arterioles, precapillary arteriole, capillaries of somatic, non-fenestrated type, postcapillary venules and venules. Aside from the contractile (typical) cardiomyocytes there is distinguished another type of the myocardium cells – conductive (atypical) cardiomyocytes, that form the heart conduction system, and the secretory cardiomyocytes.

For the first time regularities have been explained of the morphological changes in the white rat's myocardium under the prolonged effect of the opioid. In the course of injecting nalbuphine during 42 days there are observed ultrastructural changes in the myocardium and its microvasculature.

After 7 days of the experiment there were detected edemas between the bundles of cardiomyocyte myofibrils, between the neighboring cardiomyocytes, occasional destructive changes in mitochondria, a moderate edema in cytoplasm of endotheliocytes; in microvessels – platelet-erythrocytic sludges, nuclei of endotheliocytes with the signs of apoptosis; presence of cardiomyoblasts which indicates a restorative processes in the myocardium. After 14 days of the experiment there are observed growing changes in the myocardium structural organelles, specifically, there was found a vacuolar degeneration, separation of sarcolemma and myofibrils, marginal location of chromatin in the nucleus, invagination of the nuclear membrane; perivascular edema, presence of spreading masses of blood plasma in the vessels' lumen, deformation of endotheliocyte luminal surface. After 28 days it was found, that sarcolemma is disassociated, destroyed in some places, cardiomyocytes' nuclei translucent, mitochondria partially destroyed, Z-lies and M-lines destroyed, myofibrils fragmented, intercalated disks torn; pathologic folds on the luminal surface of endotheliocytes, erythrocytes with their change form and size. On the 42-nd day of the experiment there was found an expansion of the intercellular space, mosaic damages of cardiomyocytes where along with the preserved cardiomyocytes there are present destroyed sarcolemmas with villous deformation, destroyed mitochondria, Z-lines and M-lines, capillaries' walls are swollen, delaminated, endothelial contacts damaged.

By application of the optical and electron microscopy confirmed, that 3 types of mitochondria are distinguished in sarcoplasm of cardiomyocytes: type 1 mitochondria of elongated form that have a relatively large volume and a well-developed complex of cristae; type 2 mitochondria of round form that, compared with type I mitochondria have a smaller volume and a smaller number of cristae; type 3 mitochondria of a very small volume but with a great number of cristae. On the 7th day of the experiment rats of the experimental group showed a tendency towards the increase of the packing density index of all types of cardiomyocyte mitochondria. After 7 days of the experiment the white rats of the experimental group showed a tendency towards the increase of

the volume fraction of mitochondria to 24.44 (19.75; 27.84) % (the control group showed 17.44 (12.16; 25.00) %) with this index growing mainly owing to type 1 mitochondria. After 14 days of the experiment index of the volume fraction of mitochondria grew up to 26.74 (18.18; 31.87) % with the greatest growth, compared with the control group, of type 3 mitochondria index. After 28 days of the experiment destruction of mitochondria had led to a drop of the indices of the volume fraction of mitochondria to 19.48 (12.74; 28.7) %. Such a drop occurred mainly owing to the abrupt fall of the ratio of type 1 mitochondria. After 35 days the volume fraction of the type 1 and type 2 mitochondria fell to 12.5 (9.09; 15.58) % and 5.49 (2.34; 8.12) % respectively. The volume fraction of type 3 mitochondria attained its maximum during the experiment and made up 5.68 (3.23; 12.7) %.

Table 1. The volume fraction of the mitochondria (all types) of the white rat`s heart cardiomyocyte during 42 days of nalbuphine administration

Groups	Me	25%	75%	IQR	U test	p value
control	17.44	12.16	25	12.84		
7 day	24.44	19.75	27.84	8.09	140	0,078
14 day	26.74	18.18	31.87	13.69	132	0,029
21 day	21.69	15.29	36.36	21.07	169	0,21
28 day	19.48	12.74	28.7	15.96	133	0,58
35 day	16.09	12.5	23.21	10.71	157	0,90
42 day	16.13	7.97	26.82	18.85	139	0,72

Me – median, 25% - 25th percentile, 75% - 75th percentile, IQR – interquartile range, U-test – Mann-Whitney U test exact value.

Source: own study.

Tab. 2. The volume fraction of the mitochondria (type I) of the white rat`s heart cardiomyocyte during 42 days of nalbuphine administration

Groups	Me	25%	75%	IQR	U test	p value
control	10.94	6.94	16.30	9.36		
7 day	16.00	10.71	19.15	8.44	155	0,17
14 day	16.28	12.79	20.45	7.66	146	0,068

21 day	19.59	10.59	33.33	22.74	130	0,025
28 day	13.33	6.66	23.03	16.38	130	0,52
35 day	12.50	9.09	15.58	6.49	151	0,75
42 day	12.50	6.71	17.88	11.17	149	0,98

Me – median, 25% - 25th percentile, 75% - 75th percentile, IQR – interquartile range, U-test – Mann-Whitney U test exact value.

Source: own study.

Tab. 3. The volume fraction of the mitochondria (type II) of the white rat`s heart cardiomyocyte during 42 days of nalbuphine administration

Groups	**Me**	**25%**	**75%**	**IQR**	**U test**	**p value**
control	5.68	3.49	6.9	3.41		
7 day	6.74	3.57	10.59	7.02	118	0,53
14 day	7.69	4.83	10.38	5.55	111	0,17
21 day	2.69	2.06	7.35	5.29	55	0,09
28 day	5.02	3.49	7.02	3.53	76	0,51
35 day	5.49	2.34	8.12	5.77	71	0,60
42 day	6.95	2.50	10.22	7.73	67	0,80

Me – median, 25% - 25th percentile, 75% - 75th percentile, IQR – interquartile range, U-test – Mann-Whitney U test exact value.

Source: own study.

Tab. 4. The volume fraction of the mitochondria (type III) of the white rat`s heart cardiomyocyte during 42 days of nalbuphine administration.

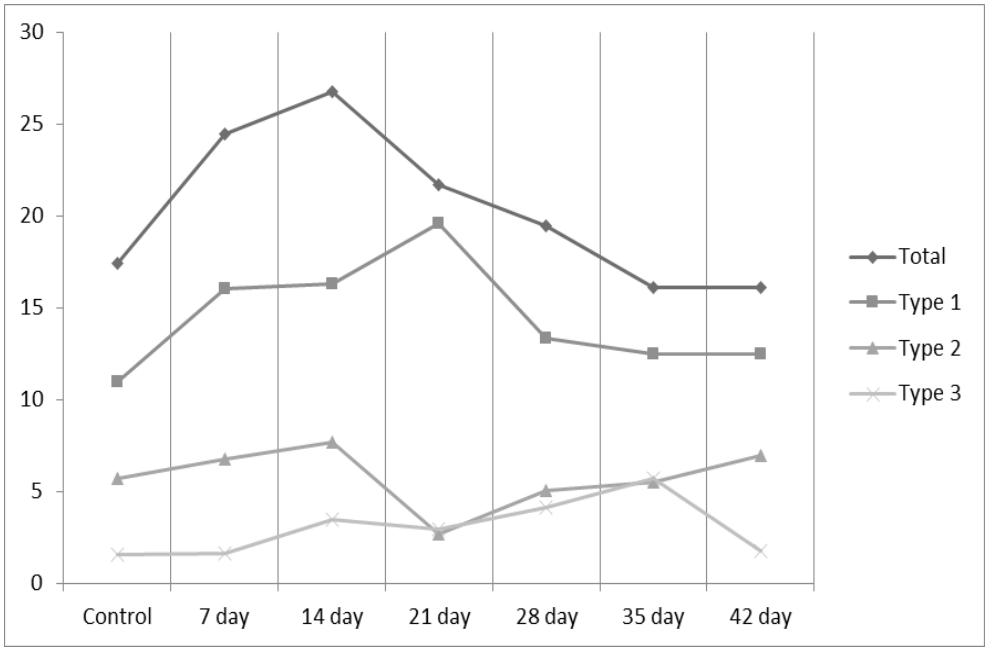
Groups	**Me**	**25%**	**75%**	**IQR**	**U test**	**p value**
control	1.58	1.28	3.49	2.21		
7 day	1.64	1.15	2.05	0.9	72	0,55
14 day	3.49	2.22	4.55	2.33	54	0,025

21 day	2.94	1.26	3.97	2.71	31	0,74
28 day	4.12	2.49	7.93	5.44	13	0,041
35 day	5.68	3.23	12.79	9.56	14	0,049
42 day	1.77	1.08	3.96	2.89	22	0,52

Me – median, 25% - 25th percentile, 75% - 75th percentile, IQR – interquartile range, U-test – Mann-Whitney U test exact value.

Source: own study.

Fig. 1. The volume fraction of the mitochondria of the white rat`s heart cardiomyocyte during 42 days of nalbuphine administration.



Source: own study.

The first signs of the disorders in the myocardium microstructure are noticeable after 7 days of nalbuphine injections to the white rats. During the following 35 days of the experiment pathological changes grow and are manifested through impairment of the well-defined structure, fragmentation of the muscle fibers, evident perivascular edema, diffuse sclerosis; the small caliber vessels are characterized by the evident hyperemia with the “sludge

syndrome" observed in the lumens of microvessels. Diffuse polymorphous infiltration is visualized of perivascular spaces by lymphocytes, macrophages and neutrophils.

Scanning electron microscopy of the white rat's heart in the course of the experiment showed changes in the relief of the interior surfaces of the heart chambers: shortened microvilli at the initial stages of the experiment and their absence, laminiform formations at the later stages. Deposits of fibrin strings, various erythrocytic figures and cholesterol crystals have been found on the interior surfaces of the heart chambers. Endotheliocytes are distributed chaotically, their form and sizes changed, endothelial contacts widened, plasmalemma of some endothelial cells forms processes with numerous microprocesses and microbubbles on their surfaces, the entire surface is covered with activated platelets and accumulations of altered erythrocytes (echinocytes, spherical, cupola-shaped, annular). Characteristic is the formation of erythrocytes and microthrombi sludges.

Having made the conclusions based on the results of our experimental study we can say that in case of drug intoxication typical is an acute disorder of microcirculation, signs of cardiosclerosis and fibrillation of atriums. Dystrophic damages of cardiomyocytes occur rather often. This permits us to speak of the narcogenic cardiomyopathy.

Discussion

Thus, the pathophysiology of these changes, caused in the rat's heart by the nalbuphine administration, can best be described as a mitochondrial and microcirculatory distress syndrome with parenchymal cells apoptosis development. The key point in the pathophysiology is the changes in mitochondria caused by POL system disturbance because of opioid administration. Opioid group's agents caused different changes in the POL system and the mechanism of these processes depending on the chemical structure of the particular drug (19-21). A critical level of mitochondria changes because of the POL system disturbance can initiate parenchymal cells apoptosis, which was revealed as the changes in cardiomyocytes nuclei morphology. The most researched compound from the opioid group is the morphine. But the experimental morphine administration more often caused rat's heart lipid dystrophy, as one of the most prominent sign, whereas nalbuphine caused swelling and the pattern of dystrophy. The local fat-storing vacuoles also observed at the ultrastructural level in a case of nalbuphine administration, but it does not lead to the well-expressed dystrophy, as in a case of morphine administration (12). Thus, these indicate that they have a different mechanism of POL system disturbance and pathology development. Moreover, we can suggest, that nalbuphine has an ability to block the peroxide because of

"scavenger" syndrome, similar to that was found in morphine in-vitro. It is only one way to explain why the increase of nalbuphine's doses does not lead to dramatic changes any at the microstructural, neither at the ultrastructural level.

The practical value of the obtained results

The obtained results of micro- and ultrastructural study of the myocardium and its microvasculature under the effect of 6 weeks long injections of the opioid are important not only for the morphologists, but for the clinicians as well. The obtained results allow us to extend the view of and resolve the issue of the effect of an opioid on the structure of the heart and its microvasculature, which creates the morphological basis for understanding pathogenesis and, subsequently, for determination of the optimal methods of diagnosing, prevention and treatment of cardiac diseases of patients, who had to use opioids for the prolonged periods of time, and that of the drug users.

Conclusion

The nalbuphine administration causes well-expressed changes on the ultra-structure of the rat heart. First of all, it's manifested as changes in the mitochondria shape, size and cristae design and numbers. Besides, there were changes in the nuclei of parenchymal cells – they becoming uneven in shape, with chromatin margination and fragmentation, what can indicate the beginning of apoptosis. Moreover, changes in the structure of microvascular flow occur – changes in the endothelial lining of heart microvessels, occlusion of the coronary vessels, erythrocytes and platelets aggregation to the luminal surface of endoteliocytes.

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