CODEN [USA]: IAJPBB ISSN: 2349-7750

INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES

http://doi.org/10.5281/zenodo.3471665

Available online at: http://www.iajps.com Research Article

CARDIOPROTECTIVE EFFECT OF METABOLIC PREPARATION IN EXPERIMENTAL DOXORUBICIN CARDIOMYOPATHY.

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Article Received: August 2019 **Accepted:** September 2019 **Published:** October 2019

Abstract:

The purpose of the study is to examine the cardioprotective properties of metabolic preparation Meldonium in case of Doxorubicin cardiomyopathy in experiment.

Materials and methods: *The research was carried out on 40 sexually mature male rabbits that received Doxorubicin four times, once a week, in a dose of 2 mg/kg with the aim of cardiomyopathy modelling. After the development of cardiomyopathy all the animals were divided into 2 groups. The rabbits in the first control group did not receive any correction measures, the animals in the second experimental group received Meldonium in a dose of 15 mg/kg intramuscularly. To identify the cardiotoxic effect of Doxorubicin and evaluate the Meldonium effect the following standard research methods were used: the electrocardiogram evaluation, biochemical blood test and morphological examination.*

Results: *The result of Doxorubicin effect on the animals' hearts in the experiment is the development of cardiomyopathy, which makes timely cardioprotective measures necessary.*

It appeared that the Meldonim injection is able to limit the development of tachycardia and the extent of myocardial damage evaluated by electrocardiogram. While in the control group the heart rate exceeded the original values in 14 days and on the 14th day the prevailing was myocardial damage of III degree.

The researched biochemical blood indicators of the experimental group reduced in the same degree as in the animal of the control group and did not differ reliably from the level of original values by the end of experiment.

The conducted left ventricular myocardium morphological examination of the animals treated with Meldonium in comparison with the animals from the control group indicated the most distinct adaptive reactions. The amount of fuchsin positive cardiomyocytes, obtained by haematoxylin-basic fuchsin-picric acid method, in field of vision of the animals from the experimental group was 6.9 ±0.6, which was 2.3 times less than in the control group, which evidences the decrease of metabolic and ischemic damage of myocardium after the Meldonium administration.

Conclusion: *The 14-day administration of Meldonium showed cardio- and cytoprotective properties, allowed to reduce the myocardial damage and the tachycardia development to a greater extent, as well as reduce the increase of biochemical blood indicators (SPGT, AST, CPK and LDH).*

Keywords: *Doxorubicin, Meldonium, cardiomyopathy, experiment, rabbits.*

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Please cite this article in press V.I. Kharina et al., ***Cardioprotective Effect Of Metabolic Preparation In Experimental Doxorubicin Cardiomyopathy.,*** *Indo Am. J. P. Sci, 2019; 06(10).*

INTRODUCTION:

Since the middle of the 1970s to the present day cardiovascular disease is the overall leader in the mortality rate of Russia [1]. Coronary heart disease is the leader among cardiovascular diseases by the number of deaths – over half of deaths (68%). Though, nowadays one of the biggest cardiological problems is congestive heart failure (CHF) due to its prevalence rate and failure rate. Thus, the survival rate of the CHF patients within 3 years after the diagnosisis is less than 50%, while the risk for sudden cardiac death is 5 times higher than among healthy people [2]. A significant role among etiological agents of CHF development lies on cardiomyopathies (CMP) and cardiomyopathies syndromes in the structure of multisystem pathology [3]. The evidence of CMP prevalence varies greatly. Thus, according to the epidemiological studies, conducted in the European part of the Russian Federation, CMP equals to 0.8% in the structure of the CHF development reasons; in the samples of European epidemiological studies it equals to 4-12% [4]. Despite the relatively small CMP prevalence, medical and social role of this group of diseases is quite important owing to the lack of clear diagnostic criteria, as well as poor survival prognosis due to the delayed diagnosis and inadequate and uncontrolled treatment in the out-patient settings [5]. Cardiomyopathy is not always the primary pathological process: it can also develop as a complication following the administration of chemotherapeutic agents and radiation therapy. In particular, the injection of anthracycline antibiotic – doxorubicin – as a treatment of a wide range of oncological diseases [6] leads to the development of the severe life-threatening cardiomyopathy [7, 8]. This fact therefore places doctors before new challenges: early diagnosis of cardiotoxicity caused by Doxorubicin and rational and effective therapy.

To date, there have been conducted plenty of works aimed at the search and study of pharmaceuticals allowing to prevent or reduce the toxicity of anthracycline antibiotics [9].

Considering that the leading role in the development of cardiotoxicity while Doxorubicin administration is connected with the activation of membrane structures lipid peroxidation and energy metabolism disorder, for the patients with Doxorubicin cardiomyopathy as a metabolic cardioprotective therapy it is logical to use medications blocking partial oxygenation of free fatty acids and regulating the energy metabolism of the cells suffered from hypoxia or ischemia.

In particular, at the present stage among the metabolic preparations Meldonium attracts strong interest [10].

Meldonium – 3-(2,2,2- trimethylhydrazinium) propionate – is the strongest reversible inhibitor of gamma-butyrobetaine hydroxylase, which facilitates the carnitine synthesis from gamma-butyrobetaine. Thus, the carnitine-dependent fatty acids transport in the mitochondrial of muscular tissues goes down and the intensity of beta-oxidation of free fatty acids diminishes. At that, only the transport of long chain fatty acids through the mitochondria membranes is limited, while the short chain ones can freely pass through and be oxidized there without the accumulation of the underoxidized fatty acids within mitochondria [11]. Also Meldonium has an antioxidant effects – it reduces the intensity of lipid peroxidation and increases the activity of endogenous antioxidant system, countering the effects of oxidative stress.

The aim of this research is to study the cardioprotective properties of Meldonium in cases of experimental Doxorubicin cardiomyopathy.

MATERIALS AND METHODS:

The experiment was conducted on 40 sexually mature male chinchilla rabbits weighing 2.8-4.2 kg in accordance with the provisions on rules of working with animals.

The animals housing in vivarium and all the experimental procedures and protocols used for this study corresponded to the rules of laboratory practice in accordance with the guideline for experimental studies [12] and the Order of the Ministry of Health and Social Development of Russia № 708N under date of 23.08.2010 "On approval of good laboratory practice regulations" (GLP), following international guidelines of European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes [ETSN 124, Strasbourg, 22.06.1998]. For experimentation the requirements of the Commission on animals treatment ethics of Russian National Committee for Bioethics and ethical norms set out in "The International Recommendations for conducting the biological studies with the use of animals" (1989) were considered. The euthanasia of the animals was carried out under obligatory ether anaesthesia.

Cardiomyopathy was pharmacologically modelled in 34 animals by Doxorubicin administration (LENS-Pharm (Russia)). Doxorubicin was administered four times, once a week in the dose of 2 mg/kg (overall accumulated dose was 8 mg/kg) intramuscularly in the marginal vein of the left ear [13]. The animal were monitored daily and weighed weekly. 6 animals – the group of untreated animals – were injected with water for injections; the dose was equivalent to the dose of Doxorubicin solution.

After Doxorubicin cardiomyopathy modeling, the left ventricle fragments were taken from 6 animals for morphological examination of the myocardium toxic damage degree. The rest of the animals were divided into 2 groups: Group 1 (10 animals) – the control group, Group 2 (10 animals) – the experimental group. As a control drug the animals from Group 1 were injected with water for injections in the volume of 2 ml/kg. In Group 2 the evaluation of effect of Meldonium administered in the dose of 15 mg/kg was carried out. The dose calculation for the experimental animals was done according to the interspecific recalculation [13]. The examined substances were administrated intramuscularly into the hip posterior surface, starting from the first day after the last dose of Doxorubicin administration, daily during 14 days at one and the same time.

To detect Doxorubicin cardiotoxic effect and evaluate Meldonium effect while Doxorubicin cardiomyopathy modelling, rabbits' biochemical blood parameters were studied and electrocardiogram (ECG) was taken. ECG was conducted on multi-channel certified electrocardiograph (Fukuda Denshi Cardimax FX-326U (Japan)), with the use of needle steel electrodes injected under the skin in the animals' limbs in I, II and III standard leads; the paper advance speed was 50 mm/s. The ECG was evaluated by the following parameters: heart rate, the length of PQ interval; the presence and the pattern of irregular heartbeat and repolarization. 3 grades of ECG change were distinguished: I – the reduction of waves R and/or T voltage, II - T-wave inversion, III – the ST-segment elevation and/or the appearance of pathologic Q-wave [14]. In the blood plasma alanine-aminotransferase (ALT), aspartate aminotransferase (AST), lactic dehydrogenase (LDH) and total creatine kinase (CK) were assessed.

To carry out comparative analyses of Meldonium effect on the myocardium state while Doxorubicin cardiomyopathy modeling, left ventricular myocardium morphological examination was conducted on the control group.

Under obligatory ether anaesthesia after Doxorubicin cardiomyopathy modelling biological material was taken from the 6 animals, the rest of the animals went through this procedure on the 14th day of Doxorubicin cardiomyopathy treatment. After the separation of heart from organs complex it was cut into fragments, containing left ventricular myocardium sized 1x1x0.5 cm. The obtained sample was preserved in 10% neutral formaline for 24 hours, after which it was embedded in paraffin using the standard methods. The tissue columns in the paraffin block were oriented so that the samples obtained allowed studying all the left ventricular myocardium thickness. The paraffin blocks were cut on the microtome to get the samples 7-10 micrometers thick. The samples were stuck on the slide plates. On the next stage the staining of the samples obtained was conducted. To observe and visualize the morphological changes, the samples obtained were stained with haematoxylin and eosin. To detect the damaged areas with ischemic and metabolic disorders, the samples were stained by haematoxylin-basic fuchsin-picric acid method [15]. The stained samples were put under cover glasses using the standard methods. The obtained histologic specimen were studied in detail under the light-optical binocular microscope, then the photographs were taken using the digital photographic attachment; then they were saved in JPG-format. Digital photo morphometry and edition was carried out on computer using Adobe Photoshop CS3 software. The morphometry was carried out on the images in 10 fields of vision at 100-fold increase. With the use of morphometry, the following parameters were measured: 1) the thickness of cardiomyocytes – the maximum lateral size of a cardiomyocyte, in

micrometers; 2) capillary luminal diameter – the size between capillary endothelial cells in transection, in micrometers. Also the correlation between the capillary diameter and the cardiomyocyte thickness was evaluated – the obtained coefficient allows to estimate the dynamics of myocardium morphological changes.

The evaluation of the metabolic state of myocardium contractile elements at the staining by haematoxylin-basic fuchsin-picric acid method was carried out by the quantitative calculation of the cardiomyocytes with the signs of fuchsinophilia on the field of vision with the use of visualization complex with "Micro-Analysis View" software package using the lens system x40 (PJSC LOMO - Microsystems). The areas of the slides under study did not intercross. To get an objective perception about the level of metabolic damages at least 100 fields of vision were analyzed in each sample section.

RESULTS AND SUBSEQUENT DISCUSSIONS:

General toxicity. During the experiment 8 rabbits that received Doxorubicin died spontaneously (2 rabbits after the dose of 4 mg/kg, 3 rabbits after the dose of 6 mg/kg and 3 rabbits after the dose of 8 mg/kg). The body mass of the animals from the untreated group did not reliably change. The animals from the control series (Doxorubicin cardiomyopathy) lost on average 350 g of their body mass, which is 10% less than the original values (table 1).

Table 1 - Average weight change of the rabbits during cardiotoxicity caused by Doxorubicin (n=22, M±m)

Groups	Original values	On 22nd day of the experiment
Untreated animals	3.6±0.08	3.6±0.06
Control series (Doxorubicin CMP)	3.8±0.06	3.4±0.07*

Note: *- p< 0,05 – the differences are significant in comparison with the original values.

The development of cardio- and cytotoxity effect of Doxorubicin was proved by such standard research methods as ECG, biochemical blood test and morphological examination.

When analyzing ECG of the animals that received Doxorubicin, the changes proving cardiotoxic effect of the preparation were found (figure 1). Thus, heart rate reduced at 14% (p<0.05) in comparison with the data obtained before the administration. 12 out of 22 animals (54%) had changes typical of myocardium damage III degree, 8 animals (37%) – II degree, and 2 animals – I degree (9%).

When analyzing untreated animals, the signs of pathological and specific changes as well as no heart rate and int. PQ changes were not revealed (figure 1).

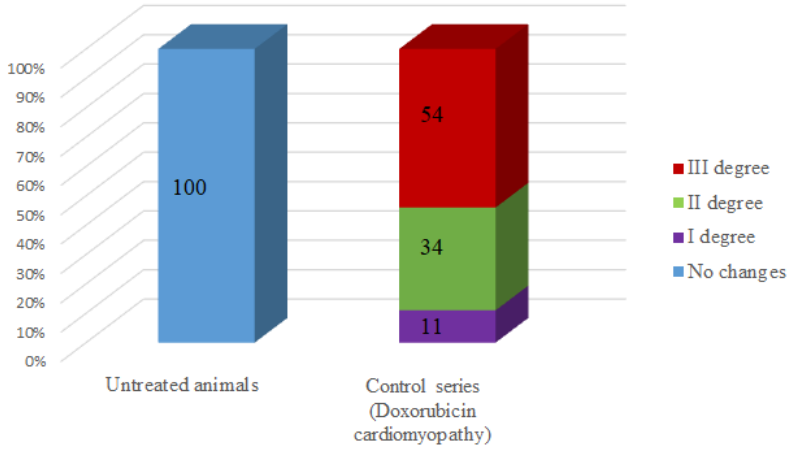


Fig 1. The rabbits' ECG rate of change at Doxorubicin cardiomyopathy (in % to the overall number of animals).

On Doxorubicin administration all the chemistry values under study increased. Thus, ALT reliably increased by 85% (p<0.05) in comparison with the original values, AST – by 168% (p<0.05), LDH – by 183% (p<0.05), CPK – by 266% (p<0.05). The

chemistry values of the untreated animals changed insignificantly (table 2).

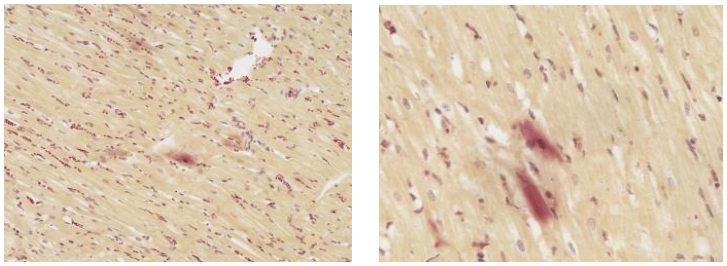
Table 2 - Blood chemistry value dynamics on Doxorubicin administration (M± m, n=22)

Values	Untreated animals		Control series (Doxorubicin CMP)	
	Original values	On 22nd day of the experiment	Original values	On 22nd day of the experiment
ALAT	42.2±2.4	40.4±3.7	41.1±2.7	76.2±4.7*
ASAT	28.7±2.1	27.8±3.4	25.6±1.7	68.8±4.2*
LDH	212±14.3	202±15.6	203.9±13.5	577.6±18.5*
CPK	263.8±15.9	254.3±10.8	284.2±14.8	1041.1±7.3*

Note: *- p< 0.05 – the differences are significant in comparison with the original values.

On the light microscopy of healthy rabbits' heart tissue characteristic features of myocardium structures were found. Endocardium and epicardial layers, covering correspondingly the inside and the outside surface of the heart, did not have any signs of pathological changes. On the myocardium morphometry, the average thickness of cardiomyocytes was 9.44±2.79 micrometers; the capillary diameter on average amounted to 5.33±1.14 micrometers. The correlation between the capillary diameter and the thickness of cardiomyocytes at healthy rabbits amounted to 0.56.

When studying the biomaterial of the healthy rabbits on staining by haematoxylin-basic fuchsin-picric acid method, cardiomyocytes with the signs of fuchsinophilia were rarely found (table 3, fig. 2). Singular cardiomyocytes with metabolic changes were detected without a visible order on the whole length of myocardium. Obviously, the detected changes of the contractive elements were reversible and constituted a transient state of cardiomyocytes metabolic status.



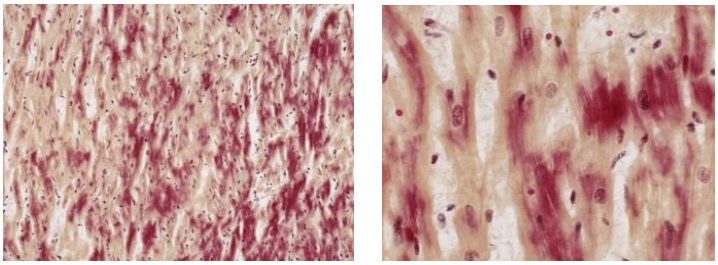
A. B.

Fig. 2. Miocardium of the untreated animals. Staining: haematoxylin-basic fuchsin-picric acid method. A – approximation x20, B - approximation x40

To prove the Doxorubicin pathological effect on myocardium, on the first day after the last dose administration left ventricular myocardium fragments were taken from some of the animals from the control series (Doxorubicin cardiomyopathy) for the morphological examination which detected significant structural changes. The myocardium was fragmented, in the area of muscular cells there were local signs of karyorhexis and karyolysis. The variability of myocardiocytes was detected, at the same time in this field of vision there were hypertrophic and atrophic cells. In cardiomyocytes with the remaining core the signs of albuminoid degeneration along with the disappearance of cross-striation were detected. There were necrosis lesions. In some fields of vision there was plasmorrhagia of vessel walls, endothelial swelling, interstitial and perivascular edema with leucocytic infiltration lesions. The morphometric data showed the following changes: the average thickness of cardiomyocytes was 8.52±2.97 micrometers, the capillary diameter on average amounted to 4.23±1.02 micrometers. The correlation between the capillary diameter to the cardiomyocyte thickness in rabbits amounted to 0.5.

On staining by haematoxylin-basic fuchsin-picric acid method in sample section of those animals that received Doxorubicin, the morphological examination showed a wide prevalence rate of metabolic and ischemic damage of myocardium. It was shown by a reliable increase of the cardiomyocytes with fuchsinophile substrate level in cytoplasm in comparison with the animals from the control group. The amount of fuchsin positive cardiomyocytes in the field of vision of the control series animals (Doxorubicin cardiomyopathy) was

8.3 times more than the untreated animals had (table 3). The formation of certain areas on which cardiomyocytes with ischemic damage formed large groups was quite often (fig. 3). A sufficiently serious degree of metabolic damage while cardiomyopathy modelling was indicated by a high intensity of cytoplasm fuchsinophilia of the contractive elements that was diffusely spread on all the structural elements of cardiomyocytes (fig. 3. b)



A. B.

Fig.3. Myocardium of the control series animals (Doxorubicin cardiomyopathy). Staining: haematoxylin-basic fuchsin-picric acid method. A – approximation x20, B - approximation x40

Table 3 - The amount of cardiomyocytes with a sign of ischemic damage (per field of vision, method - haematoxylin-basic fuchsin-picric acid staining)

Groups	The amount of cardiomyocytes with a sign of cytoplasm fuchsinophilia
Untreated animals	1.9 ±0.1
Control series (Doxorubicin CMP)	15.7 ±1.3*

Note: *- $p<0.05$ – in comparison with the animals from the control group

Thus, after the 4-time Doxorubicin administration with the accumulation dose of 8 mg/kg, on the 22nd day we detected ECG, biochemical blood indicators and morphological changes in all of the animals, which pointed at the cardiotoxic effect of the preparation.

After Doxorubicin cardiomyopathy modelling all the animals were divided into 2 groups: the experimental one and the control one. The animals from the experimental group received Meldonium as a medical treatment of cardiomyopathy.

It turned out that Meldonium administration facilitates limiting the tachycardia development. Thus, on the 7th day the heart rate was still higher than the original values by 15% ($p<0.05$), on the 14th day it lowered and did not significantly differ from the original values taken for conditional standards. While in the control group on the administration of water for injections, heart rate outnumbered the original values during 14 days (table 4).

Table 4 - Heart rate at Doxorubicin cardiomyopathy modelling in rabbits during its treatment (M± m, n=8)

	Initial values	Cardiomyopathy – day 1	Day 7	Day 14
Control group	240±7	285±15*	289±8.5*	275±12*
Experimental group	236±8.3	277.5±11*	272±14*	245±8

Note: *- $p< 0.05$ – the differences are significant in comparison with the original values.

In the animals which received Meldonium the ECG-data showing myocardium damage of I degree were prevalent both on day 7 and day 14, while myocardium damage of III degree was hardly in evidence. In the control group prevalent on day 7 was the damage of III degree, amounting at 44.5% out of overall number, on day 14 it was observed in 1/3 of the animals (fig. 4).

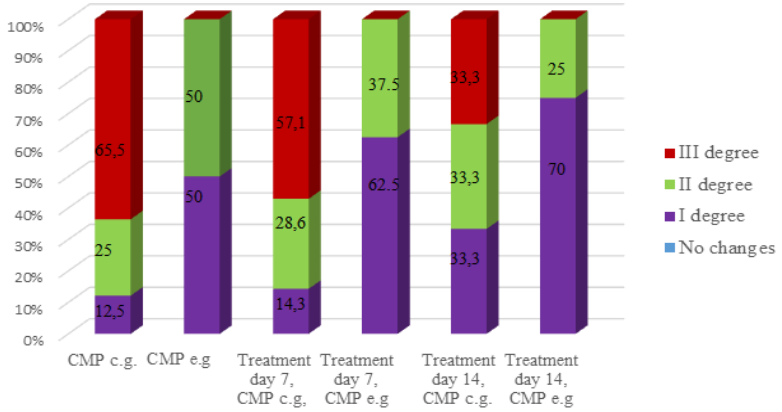


Fig. 4. The rabbits' ECG rate of change at Doxorubicin CMP modelling and during the treatment (in % to the overall number of animals in a group). Note: c.g. – control group; e.g. – experimental group.

On the 7th day of Meldonium administration to the animals with Doxorubicin cardiomyopathy the ALT and AST levels lowered at the same rate as in the animals from the control group and did not reliably differ from the original values rate. LDH and CPK in blood plasma were still on a significantly high level exceeding the rate of the healthy animals at 200% and 196% correspondingly, still they were reliably lower than the control group values rate. The further Meldonium administration resulted into lower levels of biochemical blood indicators under study and to reaching their values measured before the beginning of the experiment as well as in the control group (table 5). Due to the absence of significant differences in the dynamics of blood indicator change in the control and experimental groups, it can be concluded that their control is an uninformative evaluation method of Meldonium effectivity in the process of Doxorubicin cardiomyopathy treatment.

Table 5 - Blood chemistry value dynamics while Doxorubicin cardiomyopathy treatment (M± m)

Control group (n=8)				
B/ch values	Original values	Day 1 after Doxorubicin CMP modelling	7th day of water for injection administration	14th day of water for injection administration
ALT	41.57±7.21	76.57±6.24*	56±9.39#	52.57±6.62#
AST	25.14±3.15	68.57±5.35*	45.57±13.43*#	31.29±3.39#
LDH	209.86±24.11	578.14±24.69*	586.29±48.44*	304.86±48.44#
CPK	283.71±18.82	1055.143±24.9*	866.57±69.06*	368.86±19.47#
Experimental group (n=8)				
B/ch values	Original values	Day 1 after Doxorubicin CMP modelling	7th day of Meldonium administration	14th day of Meldonium administration
ALT	40.71±3.03	75.86±7.08*	55.14±7.03#	37.57±3.67#
AST	26.14±3.15	69±6.35*	36.71±6.93#	27.71±4.93#
LDH	197.86±3.93	577±28*	395.43±26.58*#	268.14±27.51#
CPK	284.71±12.02	1027±11.04*	557.71±37.93*#	335.86±28.94#

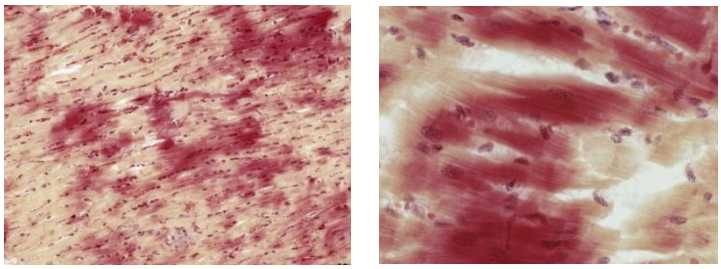
Note: *- $p<0.05$ – in comparison with the values, obtained before the injection; # - $p<0.05$ – the differences are reliable in comparison with the 1st day after the last dose of Doxorubicin administration

The conducted morphological study of left ventricular myocardium of those animals' hearts which were treated with Meldonium in comparison with the control group animals showed a significant increasing of myocardial hypertrophy, reaching its peak. It is important that concurrently the capillary diameter increases a bit. The ratio of capillary diameter to the cardiomyocytes thickness is the smallest and equals to 0.46 (table 6). Though, it must be viewed as the proof of the most expressed adaptive reactions when the conducted treatment leads not only to the structural changes administration but to the increase of the functional tissues volume. The blood flow (the capillary diameter) cannot react on the increase of myocardium volume so quickly; to evaluate these processes remote results need to be studied (months, years).

Table 6 - Morphometric values of rabbits' myocardium

Groups	Untreated animals		Doxorubicin (control series)		Control group		Experimental group	
	CMP Thickness	Capillary Diameter	CMP Thickness	CMP Thickness	Capillary Diameter	Capillary Diameter	CMP Thickness	Capillary Diameter
Min	5.14	2.57	4.5	5.59	3.17	3.02	5.59	3.17
Max	15.88	7.2	14.8	17.2	7.3	6.9	17.2	7.3
Average	9.44± 2.79	5.33± 1.14	8.52±2.97	10.76± 2.59	4.98± 0.97	4.62± 1.01	10.76± 2.59	4.98± 0.97
Coefficient (Capillary Diameter / CMP Thickness)	0.56		0.5		0.48		0.46	

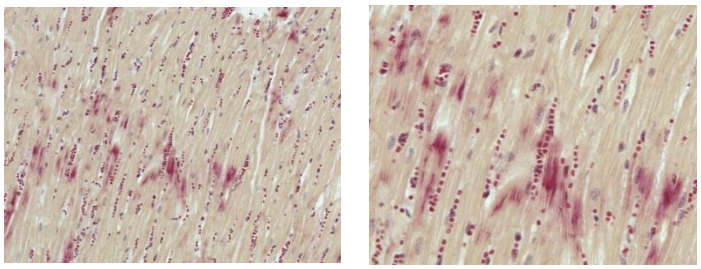
While studying control group animals' myocardium slices that were stained using the haematoxylin-basic fuchsin-picric acid method on a larger area there were a significant number of cardiomyocytes with diffusely spread intermediate lesions of moderate cytoplasm fuchsinophilia, localized mostly in the area of their cores (figure 5). Occasionally on some areas of the slices, cardiomyocytes with intensive fuchsinophilia of all the myocardium structure elements that mixed up with each other forming significant areas were detected.



A. B.

Fig. 5. Morphological pattern of left ventricular myocardium on water for injection administration to the animals with Doxorubicin CMP during 14 days. Staining: haematoxylin-basic fuchsin-picric acid method. A – approximation x20, B - approximation x40.

While studying experimental group animals' myocardium slices in some fields of vision there were small in number areas represented by cardiomyocytes with the lesions of mild or moderate cytoplasm fuchsinophilia localized mostly in the area of their cores (figure 6).



A. B.

Fig. 6. Morphological pattern of left ventricular myocardium on Meldonium administration to the animals with Doxorubicin CMP during 14 days. Staining: haematoxylin-basic fuchsin-picric acid method. A – approximation x20, B - approximation x40.

The number of fuchsin positive cardiomyocytes, obtained by haematoxylin-basic fuchsin-picric acid method staining, amounted to 6.9 ±0.6 in the field of vision of the experimental group animals, which was 3.6 times more that the amount, obtained from the untreated animals, and 2.3 lower than that in the control group (table 7), which indicates the decrease of metabolic and ischemic damage of myocardium on Meldonium administration. Thus, Meldonium administration as a Doxorubicin cardiomyopathy treatment leads to significant favorable developments in heart tissue, the enhancement of compensatory-adaptive and reparative processes.

Table 7 - The number of cardiomyocytes with the signs of ischemic damage per field of vision, method - haematoxylin-basic fuchsin-picric acid staining)

Groups	The number of cardiomyocytes with the signs of cytoplasm fuchsinophilia
Untreated animals	1.9 ±0.1
Control series (Doxorubicin CMP)	15.7 ±1.3*
Experimental group	6.9 ±0.6 *#
Control group	14.6±0.9*

Note: *- true to the healthy animals; # - true to the animals with cardiomyopathy

CONCLUSION:

Thus, the experimental Doxorubicin administration in the overall accumulation dose of 8 mg/kg causes a complex of responsive-dystrophic changes in myocardium shown as severe non-coronarogenic structural changes of left ventricular myocardium, that require timely cardioprotection to minimize the risk of cardiomyopathy development. The 14-day Meldonium administration showed cardio- and cytoprotective properties, to a greater extent allowed to reduce the myocardium damage and the tachycardia development, as well as reduce the biochemical blood indicators increase (ALT, AST, CPK and LDH).

List of symbols and Abbreviations:

ALT – alanine-aminotransferase
AST - aspartate aminotransferase
CMP - cardiomyopathies
CK – total creatine kinase
LDH - lactic dehydrogenase
ECG - electrocardiogram

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