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THE ROLE OF TNF-α IN THE PATHOGENESIS OF DOXORUBICIN CARDIOMYOPATHY

312.01. Physiology and pathophysiology

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SUMMARY

CONCEPTUAL ITEMS OF THE RESEARCH

Actuality and importance of the studded problem. Doxorubicin (Dx), as an antineoplastic remedy, imposes itself through a dichotomous pharmacological action: on the one hand, it demonstrates a notable antitumor efficiency in the treatment of patients with acute and chronic leukemia, lymphomas, sarcomas and breast cancer, and on the other hand, manifests evident cardiotoxicity, which already at a cumulative dose of 500-550 mg/m2 affects the myocardium morphofunctionally, inducing a severe heart failure imminent to dilated cardiomyopathy, also defined as doxorubicin cardiomyopathy (DxCMP) or anthracycline cardiomyopathy. Thus, in about 15% of oncology patients treated with Dx, the respective treatment is stopped due to fatal repercussions deriving from the cardiotoxicity of the drug (severe heart failure, cardiac arrhythmias, cardiac arrest). Another part of oncological patients, who do not refuse the administration of anthracycline, is forced to reduce the dosage of the drug in order to prevent or reduce the risk of rapid development of congestive heart failure (CHF).

According to the data obtained in several studies, the mortality due to CHF, which evolves within DxCMP, reaches rates up to 50% already in the first year. Moreover, the evolution of CHF can take place quite quickly, the minimum terms announced being 1-2 months [1]. Under this aspect, it is understandable that the risk of death of the oncology patient who administered Dx is twice as high, deriving from the hazard of the neoplastic process and cardiomyopathic insufficiency. Therefore, DxCMP is an exemplary paradigm of interdisciplinary objective in current medicine, denoting equal and desired valences of alertness in both oncology and cardiology, where joint research of the problem is opportunely required. Currently, the relevance of the field of Cardiology - Oncology is recognized, which records a collaboration between cardiologists and oncologists aimed at the earliest possible diagnosis of Dx cardiotoxicity and reaching the cumulative therapeutic dose of anthracycline without the eminent risk of developing DxCMP [2].

A notable step forward in this area would be marked by 2 cardinal arrangements:

1. Highlighting early predictors of Dx cardiotoxicity and, respectively, of consequent heart failure, as well as imminent pathogenetic mechanisms, which may become an important target for diagnosis as well as therapeutic influence.
2. Exploration and implementation in the oncology clinic of different feasible possibilities to attenuate Dx cardiotoxicity and the evolution of CHF, a fact that will facilitate at the same time the promotion of high cumulative doses of anthracycline in order to achieve the anti-neoplastic effect.

The clinico-experimental researchers regarding the pathophysiology of DxCMP over several decades has discovered a comprehensive pathogenetical mechanisms, as [3, 4]:

- inhibition of topoisomerase type II, DNA replication and RNA transcription, increasing the formation of free oxygen radicals, as well as the removal of histones from the nuclear chromatin;
- triggering cell apoptosis by action of the superoxide anion, responsible for the activation of caspase 3 and of "heat-shock"-25 protein;
- the excessive loading of cardiomyocytes with calcium, determined in part by the activation of slow calcium channels (voltage-depended, L-type) and the efflux of the cation from the intracellular reserves stored in the sarcoplasmic reticulum, while the pump SERCA2a (calcium ATPase) is inhibited. Excess calcium activates calpains, which mediate 2 detrimental

phenomena in the cardiomyocyte: activation of caspase 12 and titin, the latter leading to degradation of the sarcomere cytoskeleton and myocardial contractile depression;

- the inhibition, already in small concentrations, of mitochondrial respiration and beta-oxidation of fatty acids, resulting in the energy decline of the myocardium, associated with the reduction of the creatine phosphate/creatine ratio, thus forming a preconditioning for cardiomyocyte apoptosis and necrosis;
- activation of extracellular matrix metalloproteinases, especially type 2 and 9. The exaggerated degradation of collagen becomes a trigger factor for ventricular dilatation, eccentric pathological remodelling of the heart and evolution of heart failure;
- increasing the expression of endothelin 1 (ET-1) in the myocardium, thereby reducing the efficiency of the coronary Gregg phenomenon and jeopardizing the inotropic effect of the myocardium.

A mechanism inherent to Dx cardiotoxicity and which presents interest in pathophysiology of circulatory dyshomeostasis in contiguity with other pathogenetic factors (oxidative stress, activation of the sympatho-adrenergic system, endothelial and haemostasis dysfunction) is the triggering of the inflammatory response.

C. Aluise et al. (2011) reported that Dx stimulates TNF-α release by murine myocardial macrophages in an anthracycline dose-dependent manner. Intact mouse and rat myocardial cells, including cardiomyocytes, do not express TNF-α [5].

M. Argun et al. (2016) found in this context, that under the action of Dx for 2 weeks the myocardium of rodents progressively expresses TNF-α, and the level of expression of the pro-inflammatory cytokine is in direct correlation with the cumulative dose of anthracycline [6].

It is also important to mention that the expression of TNF-α under the action of Dx is also found in the wall of the coronary arteries, a fact that significantly influences the activity of the inflammatory process and the functional nativity of the endothelium and, respectively, the endothelium-dependent vascular response, thus mediating through NO and PGI2. The inflammatory damage of coronary arteries endothelium is an important mechanism in triggering and promoting coronary remodelling after mechanical stress of angioplasty, and inhibition of TNF-α, in a previous our study, it was shown to be able to ameliorate compromised coronary reactivity of the heart with diabetogenic dysfunction [7]. The TNF-α antagonist has shown, according to the dynamics of LV end-diastolic pressure, by comforting action on myocardial tolerance in the impact of ischemia-reperfusion.

The activation of non-specific inflammation it is important in the evolution of cardiovascular diseases with developing of heart failure, acting direct or indirect, by the stimulation of oxidative stress, the activation of fibroblasts and extracellular matrix metalloproteinases, the differentiation of fibroblasts into myofibroblasts mediated by the transforming growth factor beta (TGF-β), downregulation of Ca-ATP-ase of the sarcoplasmic reticulum (SERCA2a), decreases of titin phosphorylation having the detrimental effect in the lusitropic function of the myocardium, increases expression of AT1 receptors for angiotensin II (Ang II), breakdown of the connection between extracellular matrix and cardiomyocyte sarcomeres, etc. In addition, TNF-α *per se* has a depressant effect on myocardial contraction and extrinsically triggering cell apoptosis [8].

Thus, it is intelligible to research the possibility of reducing the Dx cardiotoxicity by inhibiting TNF-α, the latter being authentic through 3 maneuvers: (1) blocking the specific

receptor expressed on the cells of the cardiovascular system, (2) administering the soluble TNF-α receptor and (3) cytokine annihilation by the monoclonal antibody (mAb -TNF-α).

The aim research: Evaluation in vitro of the cardiac dysfunction mechanisms under repeated action of doxorubicin, as well as the effect of TNF-α inhibition by the administration of the monoclonal antibody (Am-TNF-α) to determine the pathogenic role of inflammation.

The objectives of the research:

1. Evaluation of the effort reactivity particularities of isolated heart under the repeated action of doxorubicin.
2. Evaluation of the coronary Gregg phenomenon under repeated action of doxorubicin.
3. Evaluation of the effect of mAb -TNF-α on the hetero- and homeometric regulation of the heart under repeated action of doxorubicin.
4. Evaluation of the effect of mAb -TNF-α on myocardial ischemia-reperfusion tolerance to repeated action of doxorubicin.
5. Evaluation of the effect of mAb -TNF-α on coronary functional reserve at repeated action of doxorubicin.

Scientific novelty and originality of research. It has been evaluated the pathogenetic mechanisms of heart failure aggravation induced by doxorubicin in different overload categories, as well as the role of TNF-α in diminishing the functional reserves of the myocardium and compromising the effort reactivity of the heart as well as of the coronary system. For the first time has been studied the benefit of systemic inflammation attenuation by administration of TNF-α antagonist on the improvement of cardiac and coronary reactivity exposed to different types of efforts in doxorubicin cardiomyopathy.

The theoretical significance of the work. The obtained results complete the pathophysiological mechanisms of doxorubicin-induced cardiac dysfunction with the new pathogenetic involvement of the pro-inflammatory cytokine, TNF-α, as well as the mechanisms of heart failure aggravation during exercise. The pathogenetic interface aimed at the interdependent input of the relaxation and isovolumic contraction phases of the heart in order to homo- and heterometric regulation of the heart in resistance and volume overload, respectively, is consolidated. It is demonstrated that emergent adaptation of the heart to hemodynamic overloads is affected by Dx, determined by reduced inotropic effect of the myocardium under the action of NE and Ang II, as well as the development of the negative inotropic effect under the action of ET-1, manifested by the reduction of left ventricle systolic pressure (LVSP) and the reduction at peak cardiac output stimulation. Conceptually, the decrease of endothelium-dependent coronary functional reserve is predicted to be important in the aggravation of heart failure under conditions of effort in conjunction with the decrease of myocardial resistance to the impact of ischemia-reperfusion. At the same time, the Vanhoutte phenomenon is not disturbed, the fact that shows the beneficial role of epoxyeicosatrienes to control of coronary perfusion in injured and impaired endothelium caused by Dx action on the heart.

The applied value of the research. The obtained results underline the pathogenetic mechanisms of heart failure induced by doxorubicin with the connotation of prognostic predictors, as well as the effect of the TNF-α antagonist to improve the functional adaptive capacity of the heart to hemodynamic and neuroendocrine stress. The isovolumic relaxation of the right heart is

highlighted as the target for estimating and correcting the heterometric regulation at volume effort, as well as the isovolumic contraction of the right heart as the target for estimating and correcting the homeometric regulation when increasing peripheral resistance. The value of +dP/dTmax and -dP/dTmax is a feasible tool to estimate the functional nativity of these phases of the cardiac cycle. Annihilation of TNF-α by administration of mAb-TNF-α has a comforting effect not only on myocardial contractile reserves but also on NO-mediated coronary Gregg phenomenon. In addition, inhibition of ETA receptors of endothelin 1 is imposed by the benefit effect regarding the compromised inotropic effect by excess of calcium.

Implementation of scientific results. The results of the study are implemented as conceptual support in the clinical cardiology of the IMPS Institute of Cardiology, as well as in the didactic program for studying the physiopathology of the cardiovascular system at the Department of Physiopathology and Clinical Physiopathology of the USMF „Nicolae Testemiţanu".

Approval of research results. The research results were presented, analysed, discussed and approved at several national and international scientific forums: 55th Congress of the Romanian Society of Cardiology, 2016; 56th Congress of the Romanian Society of Cardiology, 2016; European Society of Cardiology Congress, 2017; 57th Congress of the Romanian Society of Cardiology, 2018; European Society of Cardiology Congress, 2018; 58th Congress of the Romanian Society of Cardiology, 2019; European Society of Cardiology Congress, 2019; 6th Congress of the Cardiology Society of Moldova, 2020; 75th Anniversary Congress "Nicolae Testemiţanu" USMF, 2020; European Heart Rhythm Association Congress, 2021; Congress of the European Society of Cardiology, 2022, "Frontiers in Cardiovascular Biomedicine"; 61st Congress of the Romanian Society of Cardiology, 2022, SEC Congress "Frontiers in cardiovascular biomedicine, Budapest, 29.04.2022.

Publications related to the thesis. It have been published 33 scientific papers related to subject of the thesis, including one publication in the journal from international databases (SCOPUS, IFCiteScore2018:1.145), 8 articles in journals from the National Register of professional journals, category B, such as the *Buletinul Academiei e Științe a Moldovei* and *Moldovan Journal of Health Sciences (Revista de Științe ale Sănătății din Moldova)*, 12 abstracts in the works of national and international conferences, such as European Journal of Heart failure, Romanian Journal of Cardiology and Collection of scientific abstracts of students, residents and young researchers; 12 participations with oral and poster communications at national and international scientific forums.

The volume and structure of the thesis. The thesis is presented on 127 pages that include: annotation (in romanian, russian and english), list of abbreviations, list of figures and tables, introduction, 3 chapters, synthesis of obtained results, general conclusions and practical recommendations, 36 tables, 22 figure; bibliography of 174 titles, 2 implementation documents, information on the exploitation of the research results and the declaration regarding the responsibility assumption.

Key words: doxorubicin cardiotoxicity, TNF-α, isolated heart, hemodynamic effort, myocardial inotropism, coronary reactivity, Gregg phenomenon, Vanhoutte phenomenon

1. METHODOLOGY OF RESEARCH

1.1. General characteristic and sample volume design

The research is a preclinical, experimental one, which was realized within the institutional project in the Scientific Laboratory of Interventional Cardiology IMSP Institute of Cardiology

In order to achieve the aim and established objectives, were formed 3 batches equivalent in number, each consisting of 18 white rats of the *Ratta Albicans* species, with the same weight (200-300 mg) and age (10-12 months): The first control group consisted of intact animals; the second group – animals with doxorubicin affection and the third group – the animals treated with mAb-TNF-α on the background of doxorubicin administration.

The intact heart model was made by administering for 2 weeks of physiologic solution i/peritoneal. Doxorubicin myocardial affection imminent to doxorubicin cardiomyopathy was reproduced in white rats by administration i/p of doxorubicin (Dx) (2% solution, Ebeve Pharma, Austria) at a cumulative dose of 16 mg/kg during 2 weeks (2 injections/week with a dose of 4.0 mg/kg of one administration), the cumulative capacity of anthracycline being known (removal from the body or reduced clearance), a model also used by other researchers [6]. To assess the effect of inflammation mitigation in doxorubicin cardiotoxicity, the TNF-α antagonist (mAb-TNF-α) was administered i/peritoneally daily for 2 weeks, during the period administration of Dx in a loading dose – 5.0 mg/kg. As mAb-TNF-α was used powder of the lyophilized remedy Remikeid (Infliximab). The animals were sacrificed by euthanasia 2 weeks after the last injection of physiologic solution, Dx, and mAb-TNF-α.

In the formation of groups of laboratory animals was taken into account the "concept of the three R" (described by Russel and Burch in 1959), which involves 3 arrangements: Replacement, Reduction, Refinement.

In all 3 groups, the rats were maintained in the appropriate regime (temperature, humidity, sunlight, etc.) and standard food ration conditions with *ad libitum* access to food and water.

For the euthanasia of the animals in all 3 groups was used solution of sodium thiopental 0.4 g/kg administered i/peritoneally. After the euthanasia of laboratory animal, the ribcage was sectioned along the longitudinal line of the sternum, and taken over heart was quickly placed in cold physiological solution (temperature 0-3^{0}C) for the progressive slowing of cardiac contractions up to weak solitary cardiac contractions with a frequency of about 5 b/min.

1.2. Research methods

The experiments were performed on the isovolumic isolated heart perfusion model according to the Langendorff method and in external work mode according to the Neely-Rovetto method. To determine functional indices, the mechanical sensor connected to the computerized real-time parameter recording system (Biomedical Data Acquisition System, Bio-Shell, Australia) or the Linearcorder MARK WR3101 recorder (Hugo Sachs Electronic, Germany) was inserted into the LV cavity. Functional indices were fixed in the experimental protocol.

Isovolumic isolated heart perfusion performed according to the classic Langendorff model allows the assessment of: the myocardium tolerance to the action of global ischemia and reperfusion; of the coronary system response to the action of natural or chemical factors with vasorelaxant and vasoconstrictor properties; of Gregg coronary phenomenon; of the Vanhoutte phenomenon, which excels through the endothelium-independent vasorelaxation effect of epoxyeicosatrienes (derivatives of arachidonic acid), which is important in conditions of alteration

and dysfunction of the vascular endothelium; of the relationship between the level of coronary hypertension and the diastolic compliance of the myocardium by estimating the dynamics of the end-diastolic pressure of the LV (LVEDP) within the gradual elevation of the retrograde perfusion pressure of the coronary system, thus the impact of coronary hypertension on the diastolic stiffness of the myocardium [9].

The effort tests reproduced on the isolated heart model perfused in working mode, according to the Neely-Rovetto method, were aimed to evaluate the feasibility of hetero- and homeometric regulation in hemodynamic effort, the efficiency of the inotropic response to neuroendocrine actions, cardiac reactivity to effort, as well as the adaptation character of the heart perfused in conditions of energy deficiency [10].

The hemodynamic effort test or effort tests with volume and resistance were performed by increasing the left atrial filling pressure (LAFP) from the minimum value of 5 cm H_2O up to the maximum value of 25 cm H_2O, the reference point being the optimal filling level of 15 cm H_2O. The modification of LAFP was made on the background of constant maintenance of the pressure resistance level in the aortic estuary - 80 cm H_2O. The appreciation of the heart heterometric regulation capacity was based on the analysis of the changing character of the pump function indices together with the indices of diastolic relaxation at the reduced LAFP levels, but at the elevated levels – the myocardial contractility indices. The effort test with resistance was performed by increasing the pressure in the aortic estuary from the comfort level (80 cm H_2O) to the level of 120 cm H_2O. Within this test, the homeometric regulation capacity of the heart is estimated, the analysis of the relationship between the capacity of maintaining the adequate values of the pump function parameters and the contractile performances of the myocardium.

The evaluative test of the myocardium inotropic response was realised by perfusing natural factors capable of inducing a positive inotropic effect, such as NE, Ang II and ET-1, in different concentrations, included in the range of 10^{-7} – 10^{-5}M, on isolated heart model perfused under physiological comfort conditions (LAFP – 15 cm H_2O and aortic pressure – 80 cm H_2O). The evaluation of myocardial inotropism was based on highlighting the ratio between increased ratio of LVSP and of pumping function indices at the peak of action of these neuroendocrine factors.

At the same time, the dynamics of the diastole indices, as well as the frequency of cardiac contraction (FCC), were also estimated as an indicator of the chronotropic cardiac response.

The cardiac performance test under the energy-deprived conditions was modelled by reducing the glucose content of the perfusate from 5.5 mM up to a critical level. The estimation the dynamics of values as: cardiac output (CO), left ventricle end-diastolic pressure (LVEDP), left ventricle systolic pressure (LVSP), the maximum velocity of contraction (+dP/dTmax) and the maximum velocity of relaxation (-dP/dTmax) is opportune in the detection of expected functional changes.

Ethical considerations. The study has taken into account the international norms of medical ethics, established by the International Declaration of Animal Rights and relevant domestic documents, regarding the number of animals, their keeping and the modelling of experiments. The research project received positive approval from the Research Ethics Committee of USMF "Nicolae Testemiţanu" (protocol nr. 40, date from 27.12.2016).

Statistical data analysis. The statistical processing of the digital material obtained was realised by using Microsoft Excel and GraphPad Prism 8.0. The data obtained were calculated using descriptive statistics (mean, median, interquartile deviation, minimum and maximum,

standard deviation). The statistical significance of the results being considered when the value of $p < 0,05$. The graphic representation of the material was realised by designed box-plot graphs, bar charts and linear diagrams.

2. SYNTHESIS OF CHAPTERS

The perfusion of the isolated heart in isovolumic regime and in external work regime used as a method of researching the function of the left ventricle and the reactivity of the coronary system, which allows the estimation of the native functional reserves of the myocardium due to using of dosed application of various hemodynamic and neuroendocrine effort tests, imminent to the clinical pattern of the cardiovascular system. By modelling these effort tests, it is also opportune to evaluate with scientific precision the effects of anti-inflammatory treatment by administrating the TNF-α antagonist (monoclonal antibody, mAb-TNF-α) during the doxorubicin action period.

2.1. The basal pattern of the perfused heart in the physiological regime

In order to estimate the real regulatory capacity, we estimated the basal pattern of the perfused heart in a physiological regime, when the pressure in the left atrium (LAFP) equals with cm H_2O, and the aortic resistance – 80 cm H_2O (table 1).

Table 1. **The value of indices of the pump function of the heart (M±SD)**

Indices	**Lot**		**p**
	Control (n=9)	**Dx (n=9)**	
Aortic jet, ml/min (M±SD) Median (P25-P75) Min -Max -% vs control	21,5±2,13 22 (20-23) 18-24	12,8±1,64 13 (12-14) 9-14 40,47%	<0,001 (1,5646E-07)
Coronary flow, ml/min (M± SD) Median (P25-P75) Min-Max -% vs control	15,9±1,45 16 (15-17) 13-18	10,8±1,3 11 (10-12) 9-12 31,65%	<0,001 (0,00014)
Cardiac output, ml/min (M± SD) Median (P25-P75) Min-Max -% vs control	37,4±3,05 38 (35- 40) 32-41	23,6±2,19 24 (22-25) 20-25 36,9%	<0,001 (6,36E-08)

Doxorubicin cardiotoxicity imminent to cumulative anthracycline dose of 16 mg/kg over 2 weeks was imposed by conclusive impairment of pump function. The main index in this regard, cardiac output, was reduced compared to the control by 36.9%, a fact that was associated with the decline of the aortic jet by more than 40%. Coronary flow also showed a significant rebound equal to 31.65%. Compromised left ventricular pumping function intelligibly derives from incompetence of myocardial contractile function and diastolic relaxation.

In this context, it is also important to mention the impact of diastole (table 2). One of the most informative indices of cardiac lusitropic function, LVEDP, increased in the Dx group by 2.53 times (i.e., with 153%). LV pressure was increased not only at the end of diastole, but also in the phase connecting the end of systole with the beginning of diastole. Thus, the protodiastolic pressure was increased by 97% above the control level, a fact that is associated with the decline of

LVSP. The maximum velocity of diastolic relaxation (-dP/dT max) of the heart in doxorubicin myocardial disease is significantly impaired compared to the control prototype by 24.75%.

Table 2. **Value of diastolic relaxation indices of the heart (M±SD)**

Indices	Lot		p
	Control (n=9)	Dx (n=9)	
LVEDP, mm Hg (M±DS) Median (P25-P75) Min-Max +% vs control	4,7±0,77 4,9 (3,9- 5,2) 3,6- 5,7	11,9±0,97 11,6 (11,5-12,8) 10,5- 13 153%	<0,001 (3,92E-07)
-dP/dT max, mm Hg/sec (M±DS) Median (P25 - P75) Min - Max -% vs control	6710±182 6680 (6565-6850) 6480- 6975	5049±171 5050 (4870- 5170) 4855-5275 -24,75%	<0,001 (2,42E-11)
DS, mm Hg/ml (M±DS) Median (P25- P75) Min- Max +% vs control	29,8±4,18 32,3 (25,8 -33,4) 24,9- 34,3	62,5±3,51 60,5 (60,2- 65,3) 58,2- 67,8 +109,73%	<0,001 (6,77E-11)
LVPDP, mm Hg (M±DS) Median (P25-P75) Min-Max +% vs control	0,78±0,11 0,72 (0,71- 0,87) 0,65- 0,95	1,53±0,13 1,59 (1,44- 1,62) 1,31- 1,72 97%	<0,001 (2,02E-07)

Note: LVEDP – left ventricle end-diastolic pressure; -dP/dT max – maximal velocity of isovolumic relaxation; DS - diastolic stiffness; LVPDP – protodiastolic pressure.

Therefore, an important functional feature of the heart inherent to Dx cardiotoxicity is the disruption of contractility and isovolumic relaxation, characterized by the index of diastolic stiffness, which is dependent on the ratio between the pressure increment during diastole and the LV filling volume. Its value in the group with Dx is more than 2 times increased (+109.73%) compared to the control index.

2.2. Reactivity of the heart to volume and resistance overload in doxorubicin affection

The effort with volume and resistance represents for the heart a hemodynamic condition for triggering the intrinsic systems responsible for heterometric and homeometric regulation.

The volume overload test is an opportunity to estimate the heart's heterometric regulatory capacity, achieved by increasing the left atrial filling pressure (LAFP) from a minimum value of 5 cm H_2O to a maximum value of 25 cm H_2O (table 3).

The greater filling of the LV chamber facilitates the development of systolic pressure. However, the difference of LVSP between Dx and the control group remains significant and is attested at the rate of 27.40%. An integral index that characterizes the feasibility of heterometric regulation of the heart is cardiac work, estimated as the product of left ventricle systolic pressure and cardiac output. Its value in the volume overload test is further reduced compared to the control and notes a decline of 55.29%. In this effort test, LVEDP increases, which was more pronounced in the group with Dx, which value at the peak of the test was 190% higher compared to the control, which signifies the degree of compromised lusitropic function of the heart.

The value of diastolic stiffness increased during the increase of LAFP to higher elevations in the Dx group compared to the control by an average of 44%, so that the difference became 156%. Indices of cardiac isovolumic relaxation and contraction (-dP/dTmax and +dP/dTmax) did not increase significantly in both groups on the background of LAFP elevation, and their difference remains significant, the decline being 29.99% and 28.22%, respectively. The increased cardiac output and aortic jet in the Dx group was below the control increment demonstrating an impairment of the ability of the heart to adapt to increased volume overload. As a result, the values of these indices became 10 and 12% lower, respectively, compared to the control prototype, and their relative decline marked 40.26 and 45.25%, respectively.

Table 3. **Functional indices of the isolated heart in increased preload (M±SD)**

Indices	**Lot**		**p**
	Control (n=9)	**Dx (n=9)**	
Aortic jet, ml/min Median (P25-P75) Min –Max	30,5±2,69 31 (29-32) 26-34	16,7±2,35 (-45,25% vs control) 16 (15-19) 14-20	<0,001 1,1555E-05
Cardiac output, ml/min Median (P25-P75) Min –Max	54,4±4,4 56 (50-59) 49-60	32,5±5,6 (-40,26% vs control) 32 (29-34) 26-45	<0,001 1,07E-05
LVSP, mm Hg Median (P25-P75) Min –Max	178,5±9,2 177 (174-185) 163-192	129,6±8,25 (-27,40%) 128 (123-137) 117-139	<0,001 1,1E-05
CW, mm Hg x ml/min Median (P25-P75) Min –Max	9710±516 9680 (9525-10155) 8900-10300	4342±251 (-55,39% vs control) 4305 (4125-4600) 4040-4610	<0,001 1,22E-09
+dP/dT max, mm Hg/sec Median (P25-P75) Min –Max	8865±522 9025 (8395-9295) 8190-9490	6364±367 (-28,2% vs control) 6260 (6100-6560) 5865-6920	<0,001 4,56E-07
-dP/dT max, mm Hg/sec Median (P25-P75) Min –Max	7553±436 7360 (7295-7890) 6900-8220	5288±414 (-29,99% vs control) 5115 (4930-5625) 4835-5910	<0,001 2,13E-05
LVEDP, mm Hg Median (P25-P75) Min –Max	6,8±0,7 6,7 (6,2-7,3) 5,8-7,8	19,7±2,9 (+190% vs control) 21,2 (17,3-22,3) 15,6-22,9	<0,001 8,22E-07
DS, mm Hg/ml Median (P25-P75) Min –Max	38,5±4,3 39,6 (36,7-41,5) 31,8-43,4	98,3±6,6 (+156% vs control) 97,6 (92,4-103,6) 90,8-106,6	<0,001 5,77E-08

Note: LVSP – left ventricle systolic pressure; CW – cardiac work; +dP/dT max - maximal velocity of isovolumic contraction; -dP/dT max – maximal velocity of isovolumic relaxation; LVEDP – left ventricle end-diastolic pressure; DS - diastolic stiffness

So, the volume overload test revealed an important pathophysiological pattern of doxorubicin cardiotoxicity in a functional plan, namely: impairment of the pumping function of the heart at minimal filling of the LV due to the compromise of the isovolumic relaxation phase and impairment of the pumping function of the heart at maximum filling of LV due to increased diastolic stiffness, which limited an imminent LV filling to optimize length-force law, the important predictor being cardiac work.

The resistance effort test is an opportunity to estimate the homeometric regulation mechanism of the heart, that is achieved by increasing the pressure in the aortic chamber from the

comfort level (80 cm col.H_2O) up to 120 cm col.H_2O (table 4). The aortic jet, the main index that characterize the feasibility of isolated cardiac pump function to adapt to increased afterload, was decreased in the Dx group by 68% more compared to the control group. As a result, the index difference between groups increased from 40.47% up to 55%. The marked reduction of the aortic jet was the main cause of the decrease in cardiac output, which at the peak of the test recorded in the group with Dx only 55.82% of the control value. At the same time, the coronary flow in this test was clearly reduced compared to the control group, a fact that indicates an important aspect, special, the resistance in the coronary system during the afterload of 120 cm H_2O is lower than the pressure in the aortic chamber in both groups.

Table 4. **The values of functional indices of isolated heart in condition of increased afterload (M±SD)**

Indices	Lot		P
	Control (n=9)	Dx (n=9)	
Aortic jet, ml/min Median (P25-P75) Min-Max	15,8±2 16 (15-17) (13-19)	7,1±1,5 (-55% vs control) 7 (6-8) (5-9)	<0,001 3,92E-05
CO, ml/min Median (P25-P75) Min-Max	29,2±3,5 29 (27-32) (24-34)	16,3±2,2 (-44,18% vs control) 17 (14-18) (13-19)	<0,001 3,44E-05
LVSP, mm Hg Median (P25-P75) Min-Max	182,7±7,4 181 (179-190) (173-193)	120,3±5,2 (-34,2% vs control) 123 (116-125) (114-126)	<0,001 1,72E-08
Ind. Opie, mm Hg ×sec/1000 Median (P25-P75) Min-Max	52,6±3,6 52 (50-56) (48-58)	30,1±3,3(-42,8% vs control) 31 (27-33) (25-34)	<0,001 2,62E-06
Ind. Veragut, 1/sec Median (P25-P75) Min-Max	158,8±9,2 158 (155-165) (144-173)	110,8±7(-30,2% vs control) 114 (104-115) (103-120)	<0,001 4,61E-08
FCC,1/min Median (P25-P75) Min-Max	299±7 299 (296-305) (287-307)	250±7(-16,4% vs control) 251 (243-256) (241-260)	<0,001 1,17E-07
LVISP, mm Hg Median (P25-P75) Min-Max	85,8±5,1 87 (82-89) (78-92)	39,6±5,2(-53,9% vs control) 41 (35-44) (32-47)	<0,001 1,11E-08

Note: CO – cardiac output; LVSP – left ventricle systolic pressure; FCC – frequency of cardiac contractility; LVISP – left ventricle isotonic systolic pressure.

LV systolic pressure is the key indicator of the estimation the adaptation of the isolated heart to the increased afterload, which elevated in both groups, but the control increment was clearly superior: 39.2 mm Hg vs 14.10 mm Hg. The relative increase of the index compared to its value recorded in the physiological perfusion regime in the Dx group was double depreciated: 14 vs 28%. Thus, at the peak of the test, the LVSP difference compared to the control increased from 25.99% up to 34.2%.

The depreciation of myocardial contraction in increasing the afterload was manifested in the cardiotoxic impact of Dx also by evident reduction of isotonic systolic pressure, thus of the pressure that ensures the ejection of the perfusate in the aortic chamber. The difference at the peak of the test reached 53.9% (39.6±7.7 vs 85.8±6.5 mm Hg) and thus was correlated with the decline of the aortic jet equal to 55%. Remarkably, that rapid adaptation of the isolated heart to increased

afterload is achieved not only by increasing LVSP, but also by increasing FCC, which increased by 5 beats/min in the Dx group and by 10 beats/min in the control group under the conditions when the parameter difference before modelling test with increased afterload, was significant and constituted 39 beats/min. The degree of increase in FCC during hemodynamic effort is a true indicator of the functional systole reserve.

Due to increase of LVSP and FCC, the Opie index, which is the product of LVSP and FCC, also increased. The value of this parameter, already in the physiological infusion regime, was significantly reduced in the group with Dx, and in the resistance effort test the decline increased with 15%, causing a greater difference of 42.8% at the peak of the test: 30.1± 3.3 vs 52.6±3.6 mm Hg x min/1000. Another index, which fully characterizes the response of the isolated heart to the connotation of increasing LVSP and FCC in resistance effort, is the Veragut index, which has a relationship with the respective indices within the isovolumic contraction phase (the systolic pressure that corresponds to the maximum contraction speed). The value of the Veragut index increased in both groups, but analogously to the dynamics of LVSP, the increment of the group with Dx was clearly diminished compared to the control: 4 vs 13%. At the peak of test the difference between the groups increased up to 30.2%.

2.3. The impact of ischemia-reperfusion in doxorubicin affection of the heart

The acute ischemic impact can be reproduced *in vitro* by partially or totally cessation of coronary perfusion of the isovolumic isolated heart for a period of 30 min, using as a predictor the dynamics of LVEDP, the initial value of which was equal in both groups and calibrated at the level of 14 mm Hg. After the period of ischemia, the coronary perfusion was resumed at the initial conditions (so 80 cm col. H2O) for a duration of 45 min, during which the value of LVEDP and LVSP were dynamically monitored, the estimation points recorded at 10, 20, 30, 40 and 45 minutes. End-diastolic pressure and systolic pressure of LV are important predictors regarding the estimation of the heart's feasibility to restore the functional activity in reperfusion, which is compromised by the accumulation of excess calcium in cardiomyocytes caused by energy depletion, acidosis, oxidative stress, as well as inflammatory response. Myocardial tolerance to the action of total ischemia as well as to the action of reperfusion was conclusively affected in animals exposed to the cardiotoxic actions of Dx (figure 1).

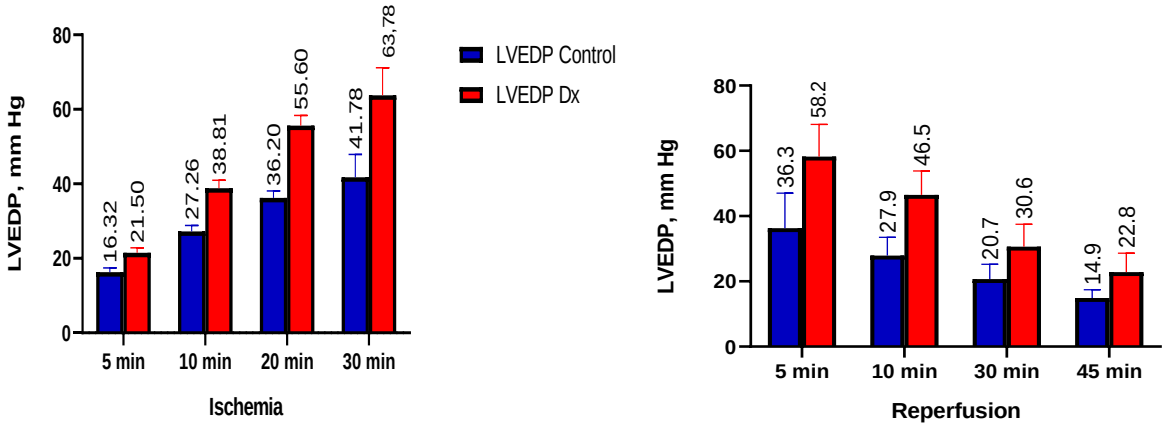

Figure 1. **The Dynamics of LVEDP (mm Hg) in ischemia and reperfusion in both groups**

Already at an early estimation of ischemic impact the LVEDP in the Dx group increased up to significantly higher values compared to the control prototype. Thus, at 5th min the difference of index between groups was 32%, and at 10th min - 43%. At the end period of ischemia (30th min) the LVEDP value increased 4.55 times compared to the pre-ischemic level (14 mm Hg), which is much more than the relative increment of control being equal with 166%. The index difference between groups increased up to 53%. Therefore, the ischemic contracture of the myocardium, estimated by the value of LVEDP, is much more in animals exposed to the cardiotoxic action of Dx, a fact that concomitantly with the dynamics of this index of the isolated heart perfused in conditions of energy depletion, modelled by excluding glucose from the Krebs solution. Under this aspect, we can appeal to the incompetence of the intrinsic control systems of calcium homeostasis in cardiomyocytes, given that the excess of the cation is the main mechanism of the ischemic contracture of the myocardium, as well as the disturbance of diastole in energy deficiency.

The doxorubicin cardiomyopathy (DCM) is characterized by a limited rate of LVSP recovery. The value of LVSP decline in DCM compared to the control established at 15th min of reperfusion increases progressively until 45th min of reperfusion: from 22 mm Hg up to 43.5 mm Hg (figure 2).

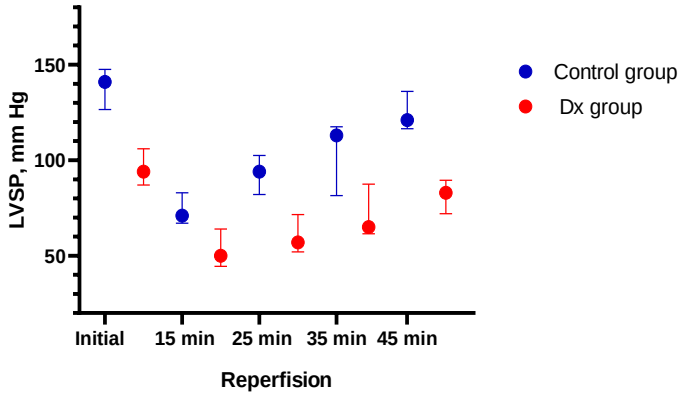

Figure 2. **The dynamics of LVSP (mm Hg) of isolated heart in reperfusion (M±SD)**

At the 45th min of reperfusion, the LVSP value remains significantly higher than the pre-ischemic value by 15% and is estimated to be below the control level by 35.2%. The latter, however, reached a level, without significant discrepancy, compared to the pre-ischemic level.

A similar difference regarding the LVSP between groups during the reperfusion period was also detected at the 25th min, when this index in the Dx group was only 67% of the control index. It is important to note that during the late reperfusion period between 25-45 min, the impact of excess calcium and the oxygen paradox (i.e., activation of oxygen stress) is promoted to the maximum.

2.4. The inotropic response of isolated heart to the action of natural trigger factors

The myocardial inotropism represents in the classical concept the property of the heart to respond to the action of different natural (or endogenous) and exogenous stimuli (eg, pharmacological remedies) by changing the force of contraction in systole in contiguity with the change in the frequency of cardiac contractions. The key intracellular element of myocardial

inotropism is represented by the "diastole-systole" calcium turnover phenomenon, so that any endogenous or exogenous stimulus that leads to an increase in the concentration of calcium in the sarcoplasm during heart systole causes a positive inotropic effect, accompanied by an increase in stroke volume and respectively cardiac output.

In the homeostatic regulation of cardiovascular system, a big role has natural stimuli which induce positive inotropic effect: catecholamines, angiotensin II (Ang II) and endothelin 1 (ET-1). These neuroendocrine factors promote the realization of physical and mental effort, help the cardiovascular reactivity, but in the pathophysiological plan they deserve special attention, due to their elevation in the circulation in conditions of heart failure (HF) development. Moreover, the severity of HF is directly related to the serum concentration of these factors, considered as a true predictor clinical and functional evolution prognosis of the patients with HF of any origin.

Under this aspect, we estimated the nature of the inotropic response of the isolated heart perfused in working regime to the administration of norepinephrine (NE), Ang II and ET-1 in a concentration of 10^{-7}M, considered in vitro conditions as physiological. To assess the feasibility of the inotropic response of the isolated heart, several functional indices of the LV were engaged, including: LVSP, cardiac output and FCC (table 5).

Table 5. **The effects of norepinephrine on the functional indices of isolated heart (M±SD)**

Indices	**Lot**	**Initial**	**NE (10^{-7} M)**
LVSP, mm Hg	Control Me (P25-P75) Min-Max	142,8±8,6 146 (135-150) 132-153	186,8±10 (+30,8% vs initial) 182 (180-196) 174-200
	Dx Me (P25-P75) Min-Max	105,7±11,4 112 (94-115) 92-119	109,7±13 (+3,8% vs initial) 118 (104-120) 88-122
p		**<0,001**	**<0,001**
FCC, 1/min	Control Me (P25-P75) Min-Max	285±8,5 289 (280-290) 268-295	309±9 (+8,4% vs initial) 313 (301-317) 295-319
	Dx Me (P25-P75) Min-Max	244±9,8 241 (237-256) 233-258	272±9,5 (+11,5% vs initial) 269 (265-282) 261-287
p		**<0,001**	**<0,001**
CO, ml/min	Control Me (P25-P75) Min-Max	36,7±2,4 36 (35-39) 33-40	45,3±3,8 (+23,4% vs initial) 47 (42-48) 40-50
	Dx Me (P25-P75) Min-Max	22,7±3,9 23 (20-25) 16-29	26,3±4 (+15,9% vs initial) 26 (24-28) 19-33
p		**<0,001**	**<0,001**

Note: CO – cardiac output; LVSP – left ventricle systolic pressure; FCC – frequency of cardiac contractility;

The analysis of the cardiac response character highlights the dissociation phenomenon of the inotropic and chronotropic effect in case of increased myocardial contractility and heart rate. Thus, the chronotropic effect was similar to the control FCC change at the peak of stimulation, the increasing of parameter being 28 bpm (11.5%) and 24 bpm (8.4%), respectively. At the same time, the increase in LVSP was much lower than the control enhancement: 4 mm Hg (3.8%) vs 44 mm Hg (30.8%).

Although the chronotropic effect of the heart to the action of NE was established in the Dx group even at a higher level compared to the control, the increase in cardiac output was much lower. As evidence, it is important to mention that CO increase at the peak of sympathetic stimulation of the heart by 3.6 ml/min (15.9%) compared to the control increment of 8.6 ml/min (23.4%). Thus, the initial difference of cardiac output from 38% increased up to 42% at the peak of stimulation and this depreciation is obviously due to the inefficiency of the inotropic response.

The increase in the circulating level of Ang II during the development of HF is closely related to the primary activation of the sympathetic-adrenergic system and the renin-angiotensin-aldosterone system. The increase in LVSP at the peak of Ang II action is less than the control enhancement (3 vs 11%), but the difference is still smaller compared to NE sympathetic stimulation of the heart (3.8 vs 30.8%). So, the positive inotropic effect of Ang II is inferior to that of the NE. The difference of LVSP at stimulation with Ang II of the heart has increased from 25.9% (initial or basal value) up to 31.2%. The chronotropic effect in both batches is weaker compared to the chronotropic effect of NE, but the difference according to the relative increase of FCC is mainly for the control pattern. The increasing rate of CO was lower than the control (10 vs 16%), which led to the increase of the difference at the peak of test from 37.8% up to 41.2% (table 6).

Table 6. **The effects of Ang II on functional indices of isolated heart (M±SD)**

Indices	**Lot**	**Initial**	**Ang II**
LVSP, mm Hg	Control Me (P25-P75) Min-Max	143,3±6,1 147 (138-148) 136-150	158,5±5,3 (+11% vs initial) 159 (155-162) 150-167
	Dx Me (P25-P75) Min-Max	106,2±9,4 112 (97-113) 95-117	109,1±12,9 (+3% vs initial) 117 (104-119) 88-122
P		**<0,001**	**<0,001**
FCC, 1/min	Control Me (P25-P75) Min-Max	286±9 289 (280-292) 268-295	300±6,5 (+5% vs initial) 299 (298-302) 288-310
	Dx Me (P25-P75) Min-Max	245±9,3 242 (237-256) 235-258	255±6,8 (+4% vs initial) 254 (250-261) 247-265
P		**<0,001**	**<0,001**
CO, ml/min	Control Me (P25-P75) Min-Max	36,8±1,8 37 (35-38) 34-39	42,7±2,6 (+16% vs initial) 43 (40-44) 39-46
	Dx Me (P25-P75) Min-Max	22,9±1,9 23 (22-24) 20-25	25,1±2,2 (+10% vs initial) 25 (24-26) 22-28
P		**<0,001**	**<0,001**

Note: Ang II - angiotensin II; CO – cardiac output; LVSP – left ventricle systolic pressure; FCC – frequency of cardiac contractility;

The inotropic effect of ET-1 on affected heart is not well known, but manifests a notable interest in context that ET-1 in present is known as one of the most powerful natural vasoconstriction factors. The action of ET-1 has manifested by distinctive particularities comparing with action of NE and Ang II (table 7).

Table 7. **The effects of ET-1 on functional indices of isolated heart (M±SD)**

Indices	Lot	Action ET-1 (10^{-7} M)	
		Initial	Stimulation
LVSP, mm Hg	Control Me (P25-P75) Min-Max	141,7±4,3 141 (138-146) 136-147	177,3±426 (+25,1% vs initial) 178 (174-180) 171-183
	Dx Me (P25-P75) Min-Max	104,9±7,6 110 (97-111) 96-112	95,4±6,6 (-9,1% vs initial) 94 (91-99) 86-107
	P	**<0,001**	**<0,001**
CO, ml/min	Control Me (P25-P75) Min-Max	36,9±3,3 38 (35-39) 31-41	42,7±4,8 (15,7% vs initial) 44 (40-45) 33-49
	Dx Me (P25-P75) Min-Max	23,6±3,9 25 (22-26) 17-28	21,5±4,1 (-9% vs initial) 23 (20-24) 15-26
	P	**<0,001**	**<0,001**
LVEDP mm Hg	Control Me (P25-P75) Min-Max	4,9±0,7 5 (4,6-5,4) 3,6-5,8	6,1±1,8 (+24,5% vs initial) 6 (4,8-8) 4-9
	Dx Me (P25-P75) Min-Max	12,5±2 12 (11-14) 10-16	17,7±3 (+41,6% vs initial) 18 (15-20) 14-22
	P	**<0,001**	**<0,001**

Note: ET-1 – endothelin 1; CO – cardiac output; LVSP – left ventricle systolic pressure; LVEDP – left ventricle end-diastolic pressure.

2.5. The coronarian phenomenon Gregg and Vanhoutte

The coronary reactivity in the classic concept defines the total response of the coronary system to the action of various factors with vasodilator or vasoconstriction action, manifested thus, by coronary dilatation and increased coronary flow or, correspondingly, coronary constriction and decreased coronary flow. Therefore, the functional entity of the coronary Gregg phenomenon determines the feasibility of coronary response dependent on the vascular endothelium and nitric oxide (NO). Atherosclerotic damage of the endothelium and NO deficiency are the main cause of impairment the coronary dilator effect of Ach, adenosine and bradykinin associated with the potentiation of the coronary constriction effect of NE, Ang II and ET-1.

At the same time, the research performed on models of isolated vessels (coronary, cerebral, femoral, etc.) proved the ability of some substances to trigger a vasodilator effect also in conditions of endothelial injury or nitric oxide synthesis blockage. The vasorelaxant effect of EET-11,12 was first investigated in several isolated vessel models by Vanhoutte, who conclusively demonstrated the ability of this arachidonic acid derivative, metabolized via cytochrome P450, to relax the muscle media despite of endothelial injury and dysfunction of it. Thus, this phenomenon of great physiopathological consideration, especially for the brain and heart, is called the Vanhoutte phenomenon.

The main factor in perfusion control with essential action is considered acetylcholine. On the isovolumic isolated heart model perfused without recirculation at an aortic pressure of 80 cm col.H2O we estimated coronary flow (CF) and coronary functional reserve (CFR) at the peak of Ach actions in the concentration from 10^{-7} M up to 10^{-5} M (table 8).

Table 8. **The coronary flow (ml/min) and CFR (%) at the action of acetylcholine (M±SD)**

Lot	CF basal	CF (ml/min) at the action of acetylcholine (M)		
		10^{-7}	10^{-6}	10^{-5}
Control Me (P25-P75) Min-Max	13,4±0,7 13,1 (12,9-14) 12,5-14,4	14,9±0,4 15 (14,7-15,2) 14,2-15,5 CFR=11,19%	16,5±0,7 16,7 (16,4-17) 15,1-17,2 CFR =23,13%	17,3±0,8 17,5 (17-17,8) 16,1-18,4 CFR =29,10%
Dx Me (P25-P75) Min-Max	12,7±0,7 12,8 (12,3-13) 11,6-13,8	13,2±0,7 13,5 (13-13,6) 12-14,2 CFR =3,94%	14,2±0,8 14,2 (13,9-14,9) 12,7-15 CFR =11,81%	14,8±0,9 14,9 (14,2-15,6) 13,4-16 CFR =16,54%
p	0,063909	<0,001	<0,001	0,00013

Note: CF – coronary flow; CFR – coronary functional reserve; Me – median; P25-P75 – interval between percentile 25 and 75

So, the cardiotoxicity of doxorubicin also is manifested by the impairment of endothelium-dependent coronary reactivity, proven on the retrograde perfusion model of the isolated heart, which does not realise the external work to the action of Ach. This evidence is important, since the constitutive action of Ach is the main landmark of the Gregg phenomenon.

The increase in coronary perfusion during the hemodynamic or neuroendocrine effort demands is ensured by metabolic factors such as adenosine (Ad) and bradykinin (Bk), which derive respectively from the kinin-kallikrein system and from the metabolism of ATP molecules, and their coronary dilator effect is not entirely dependent on the endothelium, therefore on NO. Remarkably, that the CFR imminent to the action of Ad and Bk in the concentration of 10^{-5}M is higher compared to the index detected in the test with Ach: 32.83% and 30.6% vs 29.1% (table 9).

Table 9. **Coronary flow (ml/min) and CFR (%) to the action of adenosine and bradykinin (M±SD)**

Lot	Action of adenosine (mol)		Action of bradykinin (mol)	
	10^{-7}	**10^{-5}**	**10^{-7}**	**10^{-5}**
Control Me (P25-P75) Min-Max	14,5±1,2 15 (13,6-15,6) 12,8-16 CFR=8,2%	17,8±2,2 18,6 (16-19) 14-21 CFR =32,8%	14,6±1,6 15 (13-16) 12,6-16,9 CFR =8,96%	17,5±1,5 17,8 (17-18) 15-20 CRF =30,6%
Dx Me (P25-P75) Min-Max	13,4±0,9 13,5 (12,6-14) 12-14,9 CFR =5,5%	14,5±1,8 14,4 (13,1-15,4) 12,3-18 CFR =14,2%	13,5±0,97 13,2 (12,8-14) 12,4-15 CFR =6,3%	14,4±1,7 14 (13,5-15) 12,7-18 CRF =13,4%
p	0,11912	<0,001	0,16019	0,0024

Note: CFR – coronary functional reserve; Me – median; P25-P75 – interval between percentile 25 and 75

In concentration of 10^{-7} M the coronary functional reserve is superior in conditions of parasympathetic stimulation of the heart. In group with doxorubicin the CFR attributed to the action of bradykinin in concentration of 10^{-5} M is significant reduced comparing with the control group by 56.25%, but to the action of Ad the decline had constituted 56.84%. Taking in account that Bk and Ad are the main metabolic factors that determine the increasing coronary flow, necessary for the increased functional activity of myocardium, we can admit that in cardiac doxorubicin disorder the depreciation of CFR at their action, limits the functional reserve of myocardium in hemodynamic and neuroendocrine effort.

The main significance is that the Vanhoutte phenomenon is not impaired in doxorubicin disorder of the heart, which is based on the production of H_2O_2 and EET-11,12 in the mammalian heart, the metabolites of arachidonic acid, with a coronary dilator effect mediated by the hyperpolarization mechanism. So, this phenomenon represents a relevant possibility to compensate the compromised endothelium-dependent Gregg coronary phenomenon in Dx cardiotoxicity. The coronary dilator effect manifested by regulating the coronary perfusion of the affected heart was also confirmed by the action of hydrogen peroxide and epoxyeicosatrienes 11,12 in 2 concentrations, 10^{-6} M and 10^{-5} M (table 10).

Table 10. **Coronary functional reserve to the action of H_2O_2 and EET-11,12 (M±SD)**

Lot	H_2O_2 (mol)		EET-11,12 (mol)	
	10^{-6}	10^{-5}	10^{-6}	10^{-5}
CFR (%) Control	14,9±1	15,7±2,2	14,1±0,7	14,7±1
Me (P:25-75)	15 (14-16)	15,2(14,2-16)	14,2 (14-14,8)	14,7 (14,4-15)
Min-Max	13,8-16,2	14-21	13-15	13,2-16,2
CFR (%) Dx	15,7±1,2	16,2±0,9	14,3±1	15,1±1,3
Me (P:25-75)	15,2 (15-16,4)	16 (16-17)	14 (13,6-15)	14,7 (14-16)
Min-Max	14,6-18	15-17	13-16	13,5-17
p	**0,06361**	**0,44361**	**0,789**	**0,54433**

Note: CFR – coronary functional reserve H_2O_2 – hydrogen peroxide; EET-11,12 – epoxyeicosatrienes; Me-median; P:25-75 – interval between percentile 25 and 75.

The crucial detected phenomenon consists on the fact that the CFR in the Dx group to the action of H_2O_2 or EET-11,12 does not differ significantly from the control index. Moreover, at both concentrations of agents, the CFR it is even higher than the control value by up to 6%. The Vanhoutte phenomenon projects not only on the beneficial vascular effects, but also has important tissue connotations. Thus, epoxyeicosatrienes are biologically active substances with antioxidant, antiapoptotic, cytoprotective, etc. Under this aspect, it is important to evaluate the tolerance of the myocardium to the ischemia-reperfusion impact, when the isolated heart is pre-treated with EET-11,12 for 60 sec, which led in the group with Dx to reduction of the LVEDP value, especially important in late period (figure 3).

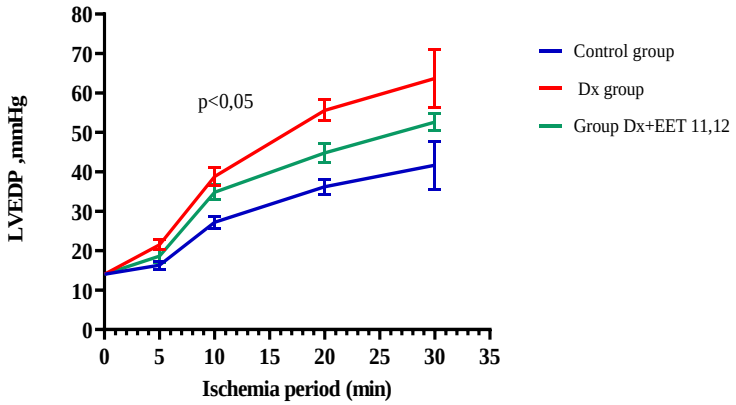

Figure 3. **The dynamics of LVEDP in period of ischemia in condition of pre-treated heart with EET-11,12**

Thus, at 20th min of ischemia LVEDP is estimated to be significantly decreased by 19.43% compared to the control index: 44.8±3.7 vs 55.6±2.7 mm Hg, but still remains significantly higher than the control value by 24%: 44.8±3.7 vs 36.2±1.9 mm Hg. The anti-ischemic effect of epoxyeicosatrienes is an important aspect of their cytoprotective and antioxidant effects. Remarkably, that also within the postconditioning of the isovolumic isolated heart with EET-11,12 the restoration of the lusitropic function of the heart was notably improved during the reperfusion period (figure 4).

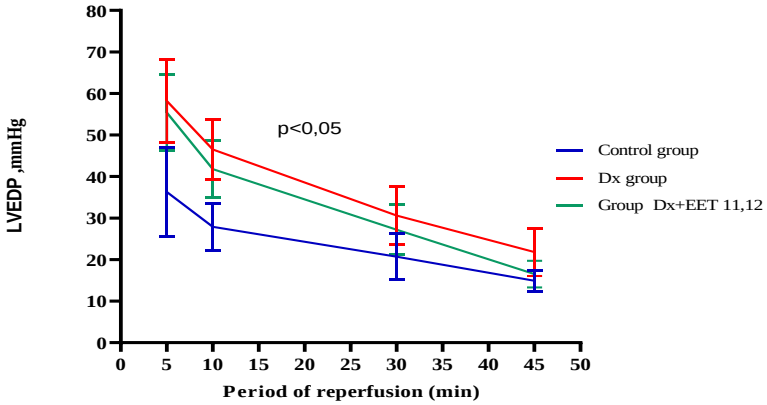

Figure 4. **The effects of postconditioning the isolated heart with EET-11,12 on the LVEDP restoration in period of reperfusion**

The correction of the coronary circuit in condition of EET-11,12 infusion in the concentration of 10^{-6} M was imposed by a more conclusive restoration of the LVEDP value, which at all time points of estimation was below the level attested in the group with Dx without postconditioning.

The evidence obtained certifies the benefit of epoxyeicosatrienes on the tolerance of the heart affected by doxorubicin to the ischemia-reperfusion impact and, at the same time, the role of the coronary myocyte hyperpolarization mechanism in limiting the ischemic contracture of the myocardium, as well as in improving diastole during the period of partial or total recovery of coronary flow.

2.6. The evaluation *in vitro* of the TNF-α antagonist effects of on the doxorubicin cardiotoxicity

Any injury to the myocardium triggers a local and sustained inflammatory response, which directly and indirectly influences the prognosis of HF evolution. At the same time, myocardial injury secondary to the action of doxorubicin induces different patterns of cell death, such as autophagy, pyroptosis and necrosis, which have notable pro-inflammatory actions. Basic research has demonstrated that rat myocardium begins to express TNF-α in proportion to the administered dose of Dx, which justifies the plausible effects of the TNF-α antagonist, the specific monoclonal antibody (mAb-TNF-α) on the reactivity of the heart and coronary system, being affected by cardiotoxic impact of doxorubicin. In the pathogenetic interface of the myocardial inflammatory response, the TNF-α has a leading role along with other pro-inflammatory cytokines, such as IL-1β, IL-8, IL-18, etc.

To highlight and confirm the relevance of the inflammatory mechanism in the evolution of Dx cardiotoxicity in another group of rats, we administered mAb-TNF-α daily concomitantly with doxorubicin action for 2 weeks (single dose 5.0 mg/kg). In both groups (Dx group and Dx+mAb-TNF-α group) animals were sacrificed after 10 days of the last injection (cumulative effect

characteristic for anthracyclines). To demonstrate the effects of mAb-TNF-α on the isolated heart, we used the same experimental protocol, especially the effort tests aimed to show the cardiac reactivity and coronary system reactivity, in which the functional particularities of Dx cardiotoxicity were conclusively highlighted.

The action of mAb-TNF-α during the development of doxorubicin damage of the myocardium was manifested by improving the pump function and contractility of the isolated heart perfused in the physiological working regime of the LV filling and the pressure in the aortic cavity (table 11).

Table 11. **The functional pump indices of isolated heart in working physiologic regimen (M±SD)**

Indices	**Groups**					
	(n=9) Control	**(n=9) Dx**	**(n=9) Dx+ Am-TNF-α**	**p1**	**p2**	**p3**
AJ, ml/min Me P25-P75 Min-Max	21,5±2,1 22 20-23 18-24	12,8±1,6 13 12-14 9-14	16,9±1,0 17,4 16,2-17,7 15,2-18	<0,001	<0,001	<0,001
CO, ml/min Me P25-P75 Min-Max	37,4±3 39 35-40 32-41	23,6±2,2 24 22-25 20-26	29,8±1,9 30,9 28,5-31 26-31	<0,001	<0,001	<0,001
LVSP, mm Hg Me P25-P75 Min-Max	143,5±3,7 143,4 142-145 138-149	106,2±5,7 106 101-111 100-114	129,3±4,4 129 125-133 124-137	<0,001	<0,001	<0,001
FCC, 1/min Me P25-P75 Min-Max	289±10 288 282-298 274-300	245±12 250 232-252 228-259	266±8 268 259-273 255-278	<0,001	<0,001	<0,001
+dP/dT max, Me P25-P75 Min-Max	8565±239 8500 8400-8680 8245-9010	6320±297 6325 6090-6540 5800-6690	7220±245 7400 7040-7465 6900-7480	<0,001	<0,001	<0,001

Note: AJ- aortic jet; CO – cardiac output; LVSP – left ventricle systolic pressure; FCC- frequency of cardiac contractility; +dP/dT max – maximal velocity of isotonic contraction; p1 – significance of discrepancy Dx vs control; p2 –significance of discrepancy Dx+mAb-TNF-α vs Dx; p3 - significance of discrepancy Dx+mAb-TNF-α vs control

A key functional benefit of mAb-TNF-α action is the significant increase of pump function indices: aortic jet by 32% and cardiac output by 27%. However, the value of these parameters remains decreased compared to the reference value. Remarkably, LVSP also increased by 22% compared to the group with Dx.

The group treated with mAb-TNF-α also demonstrated a considerable improvement in diastolic relaxation, due to decrease in end-diastolic pressure by 38.1% and diastolic stiffness by 34.24%, thereby facilitating LV filling and the realization of the Frank-Starling mechanism. It is important to note that the basic component of diastole, the isovolumic relaxation, improved significantly, given the significant increase by 25% of the -dP/dT max value to a non-significant difference compared to the control, equal to 6.41% on average (table 12).

Table 12. **Functional relaxation indices of isolated heart in working physiologic regimen (M±SD)**

Indices	Groups					
	(n=9) Control	(n=9) Dx	(n=9) Dx+ mAb-TNF-α	p1	p2	p3
LVEDP, Me P25-P75 Min-Max	4,7±0,8 4,9 3,9-5,2 3,6-5,7	11,9±0,9 11,6 11,5-2,8 10,5-13	7,8±0,8 7,4 7,3-8,5 6,6-9,1	<0,001	<0,001	<0,001
DS, mm Hg/ml Me P25-P75 Min-Max	29,8±4,2 32,3 25,8-33,4 24,9-34,3	62,5±4,4 60,5 60,2-65,3 58,2-67,8	41,1±2,1 41,2 39,4-42,7 38,6-44,2	<0,001	<0,001	<0,001
-dP/dT max, Me P25-P75 Min-Max	6710±182 6680 6565-6850 6480-6975	5045±168 5050 4870-5710 4855-5275	6280±171 6305 6215-6400 5980-6475	<0,001	<0,001	<0,001

Note: LVEDP – left ventricle end-diastolic pressure; DS – diastolic stiffnes; -dP/dT max – maximal velocity of isotonic relaxation; p1 – significance of discrepancy Dx vs control; p2 –significance of discrepancy Dx+mAb-TNF-α vs Dx; p3 - significance of discrepancy Dx+mAb-TNF-α vs control

Another important side of the action of mAb-TNF-α is the increase in the capacity of the LV to develop pressure in effort with resistance, reproduced by the elevation of the pressure in the aortic cavity to the level of 120 cm col.H2O (table 13).

Table 13. **Functional indices of isolated heart in effort with resistance (M±SD)**

Indices	Control (n=9)	Dx (n=9)	Dx+mAb-TNF-α (n=9)	p1	p2	p3
AJ, ml/min Me P25-P75 Min-Max	15,8±2 15 15-17 13-19	7,1±1,5 7 6-8 5-9	10,6±2,3 12 9-12 7-13	<0,001	0,0046	0,0022
CO, ml/min Me P25-P75 Min-Max	29,2±3,5 29 27-32 24-34	16,3±2,2 17 14-18 13-19	21,8±3,4 22 19-24 17-26	<0,001	0,0069	0,0004
LVSP, mmHg Me P25-P75 Min-Max	182±7 181 179-190 171-193	120±5 123 116-125 114-126	146±9 142 142-151 131-156	<0,001	<0,001	<0,001
LVISP, mmHg Me P25-P75 Min-Max	86±5 87 82-89 78-92	40±5 41 35-44 32-47	56±5 59 51-60 48-61	<0,001	<0,001	<0,001
+dP/dTmax, mmHg/sec Me P25-P75 Min-Max	9887±1039 9865 8990-10635 8865-11756	6835±672 6660 6265-7300 5900-7850	7754±503 7800 7655-8105 6930-8275	<0,001	0,0223	0,0012

Note: AJ- aortic jet; CO – cardiac output; LVSP – left ventricle systolic pressure; LVISP – left ventricle isotonic systolic pressure; +dP/dT max – maximal velocity of isotonic contraction; p1 – significance of discrepancy Dx vs control; p2 –significance of discrepancy Dx+mAb-TNF-α vs Dx; p3 - significance of discrepancy Dx+mAb-TNF-α vs control.

The maxim systolic pressure of LV and isotonic systolic pressure are basic indices for estimating the feasibility of cardiac adaptation to resistance effort. Under the action of mAb-TNF-α the value of them increased significantly by 16% and 54%, respectively. Special attention deserves the significant increase by 14% of the +dP/dT max index compared to the reference value attested in the group with Dx, a fact that shows the activation of the isovolumic contraction of the heart, an important effect also in the context of that the increased expression of TNF- α exerts a cardiodepressive effect on the myocardium. As a result, the attenuation of inflammation has conditioned the significant increase in aortic jet value and cardiac output in the resistance effort test by 42% and 34%, respectively.

The adaptation of the heart to this maximal resistance effort, recognized as the "Achilles' heel" requires optimal achievements of: (1) the relationship coronary pressure – end-diastolic pressure, (2) the relationship diastolic stiffness – LV filling volume and (3) end-diastolic volume – end-diastolic pressure. The action of mAb-TNF-α led to a significant increase in coronary flow due to a significantly lower elevation of PTDVS.

As evidence, it should be reported that the coronary flow in the group receiving mAb-TNF-α during the Dx action reached a 30% higher value inn condition of clamping aorta compared to the index of the Dx group: 21.4±2.1 vs 16, 5±1.7 ml/min. The reducing rate of CF compared to control group was decreased at the peak of the test by 41.87%: from 41.49% (Dx group) up to 24.12% (Dx + mAb-TNF-α group).

The increase of the volumetric velocity of coronary flow leads to the elevation of LVEDP, a characteristic evidence of the relationship coronary pressure – end-diastolic pressure which was attested in all 3 groups. It should be noted that in condition of aortic clamping, the LVEDP elevation rate in the Dx group equal to 61%, was reduced by 23% in the Dx+mAb-TNF-α group, which was 47%. The average value of LVEDP became 21.65% lower: 15.2 vs 19.4 mm Hg.

The results obtained in this effort test applied on the isolated heart perfused in working regimen demonstrate the benefit of mAb-TNF-α on diastole, which was also confirmed on the isolated isovolumic heart in the test with increasing the coronary pressure from 80 up to 120 cm col. H_2O (figure 5).

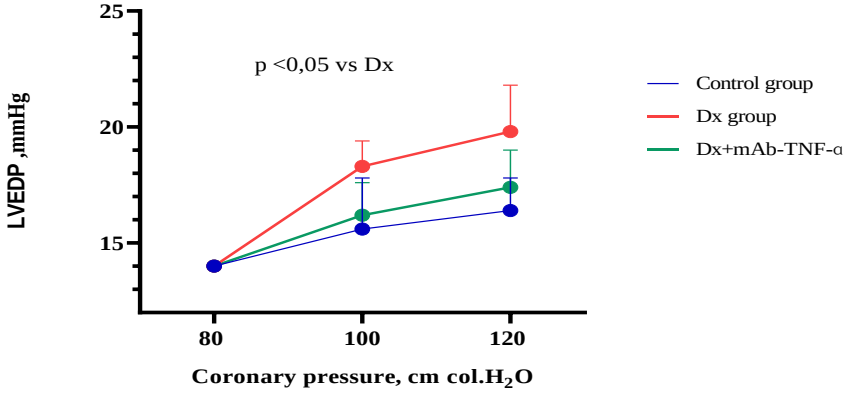

Figure 5. **Relationship coronary pressure – end-diastolic pressure of isolated isovolumic heart**

The administration of mAb-TNF-α was imposed by the notable reduction of the LVEDP elevation slope of the isovolumic isolated heart in condition of gradual increase in coronary

pressure. It should be noted that before reproducing the effort test, the LV end-diastolic pressure was calibrated at the same level, 14 mm Hg, in all 3 groups.

The LVEDP in the Dx group increased by 42%: from 14 to 19.8±1.5 mm Hg. Inhibition of inflammation by mAb-TNF-α reduced this rate up to 12.4%, due to elevation of LVEDP at the level of coronary pressure of 120 cm col.H_2O up to 17.4±1.3 mm Hg.

So, we can highlight the consecutiveness of the pathophysiological events: the increase in the volumetric velocity of the coronary flow and, respectively, of the coronary pressure leads to the elevation of LVEDP, which subsequently results in the increase of the diastolic stiffness of the myocardium which limits the coronary perfusion. Diastolic stiffness is a pathophysiological entity that characterizes the feasibility of diastole, and the LVEDP level is the quantification value of the severity of the stiffness and, therefore, of the disturbance of the lusitropic function of the heart, regarding the filling of the left ventricular cavity.

An important criterion for *in vitro* assessment of the functional nativity of diastole is the relationship diastolic volume – diastolic pressure. This relationship is compromised, as demonstrated above in the Dx group, according to LVEDP dynamics when left atrial filling volume increases. The action of mAb-TNF-α notably improved the diastolic volume – diastolic pressure relationship, given that the elevation rate of LVEDP was lower than it dynamics in the groups with Dx (Figure 6).

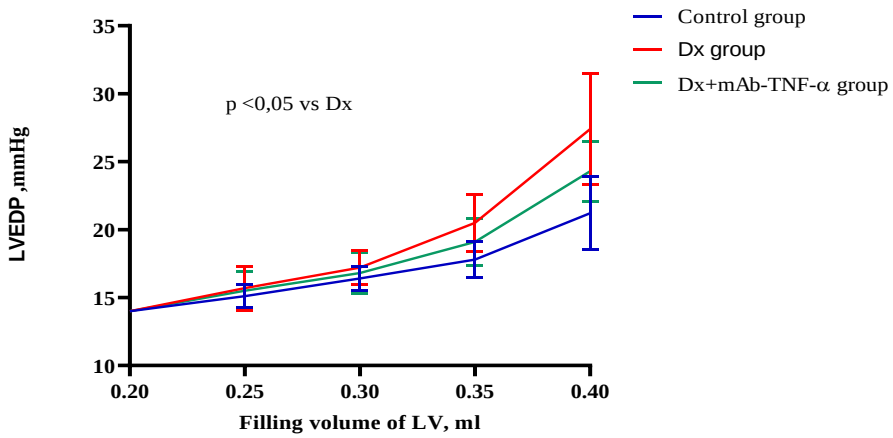

Figure 6. **The LVEDP dynamics in condition of gradual increasing the filling volume of LV**

The increasing rate of LVEDP in condition of mAb-TNF-α action was reduced by 22.9% compared to this index in the Dx group based on the increasing the LV filling volume from 0.2 up to 0.4 ml: 74 vs 96%. At the end point of estimation (filling volume equal with 0.4 ml) the decline of LVEDP became significant, marking an average of 11.3%: 24.3±1.4 vs 27.4±1.6 mm Hg. However, compared to the reference index, the difference remains significant, attested at rate of 15%.

A more limited increasing of LVEDP secondary to the induced enhancement of LV volume during retrograde perfusion of the isolated heart indicates decreased myocardial diastolic stiffness. It is important to mention that this mechanism is responsible for reduction of the volumetric velocity of coronary flow, as the inverse relationship also is valid, which highlights the dependence of myocardial perfusion on diastolic compliance.

The attenuation of inflammation by administration of mAb-TNF-α has decreased the decline of CF in condition of gradual increasing of filling volume of left ventricle (figure 7).

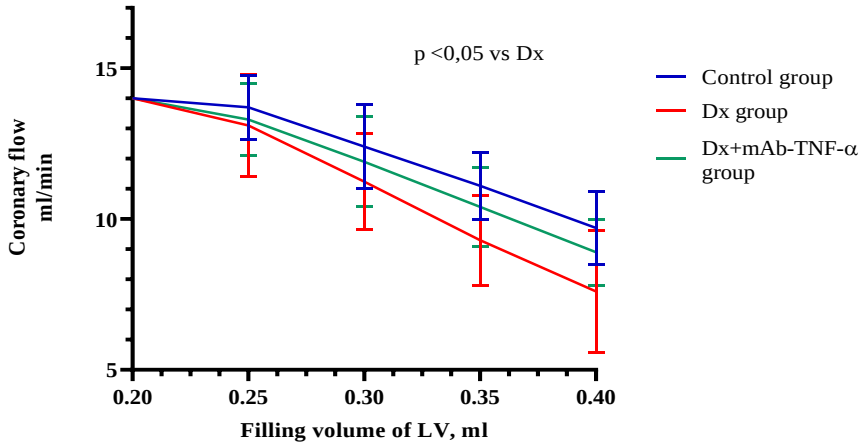

Figure 7. **The coronary flow dynamics in condition of gradual increasing of left ventricle filling volume**

In all groups, the initial value of the coronary flow of the isolated heart retrogradely perfused through the aorta was, as in the case of the LVEDP value, equal in all groups, measuring on average 14 ml/min. The depreciation of CF in the Dx group during the doubling of the LV filling volume (from 0.2 up to 0.4 ml) was 45.72%. Under the action of mAb-TNF-α, the reduction rate of CF has decreased by 20.32% and stabilized at a rate of 36.43%, and the absolute value of the index was significantly higher by 18% compared to the index in the Dx group: 8.9±0.5 vs. 7.6±0.5 ml/min.

The lusitropic regulatory system of the heart is a component of the myocardial interface that orchestrates its tolerance to ischemia-reperfusion and engages events related to the control of energy metabolism, calcium paradox and oxidative stress. The myocardial tolerance to ischemia-reperfusion action in the cardiotoxic impact of doxorubicin is considerably impaired, a distinct phenomenon, taking in consideration the inflammatory preconditioning imminent to the myocardial anthracycline injury. Administration of mAb-TNF-α during the cardiotoxic action of Dx increased myocardial resistance to the impact of ischemia and reperfusion.

Thus, the LVEDP value was significantly lower, compared to the index in the group with Dx, throughout the ischemia period (figure 8). Against the background of mAb-TNF-α action, the LVEDP value estimated during the period of ischemia in DxCMP was progressively reduced up to a decline of 19.3% established at min 30 of ischemia.

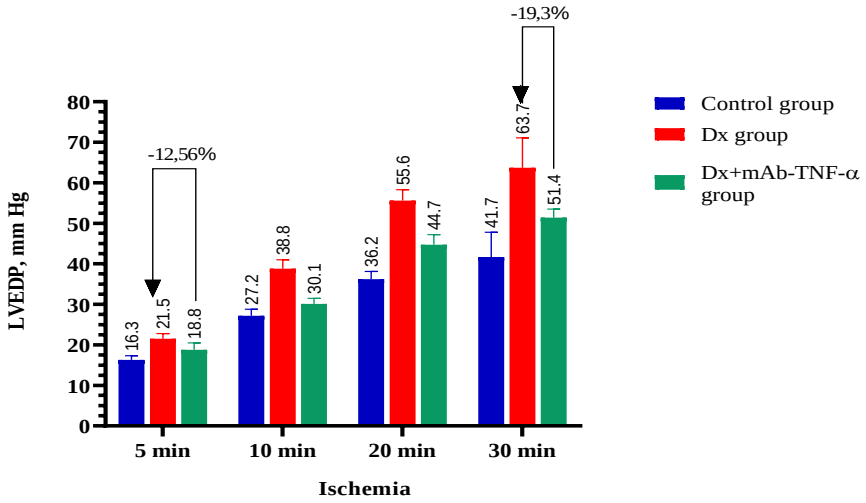

Figure 8. **The effect of mAb-TNF-α on ischemic tolerance of the myocardium estimated by the LVEDP dynamics during ischemia**

The reduction of myocardial ischemic contracture estimated by LVEDP dynamics indicates more conclusive functional and metabolic reserves of the myocardium under the action of mAb-TNF-α and this effect was also confirmed during the reperfusion period, which is characterized by the impact of oxygen and calcium.

The attenuation of inflammation by mAb-TNF-α administration excelled through a more obvious restoration of diastolic relaxation of the isovolumic isolated heart under conditions of coronary flow recovery (Figure 9). Remarkably, at the end of the reperfusion period the LVEDP value is estimated to be 28.5% lower than in Dx group (p=0.00743) and does not differ significantly from the control

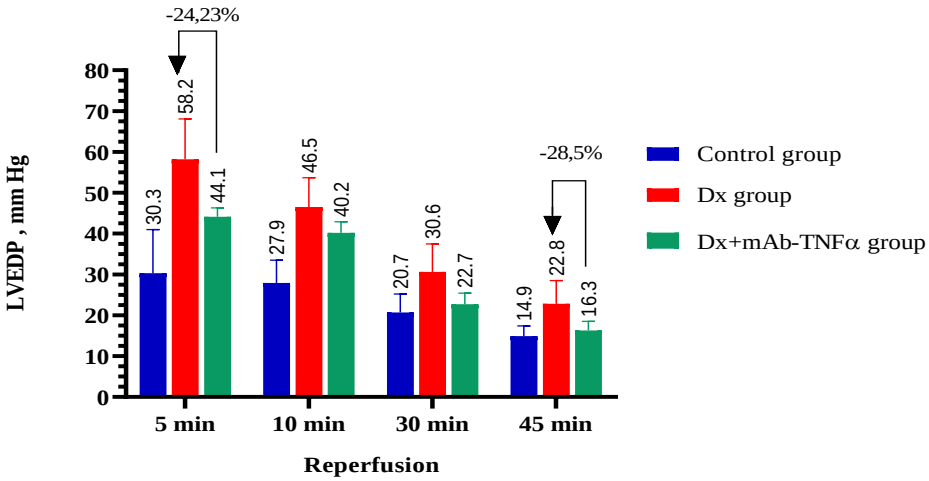

Figure 9. **The effect of mAb-TNF-α on the ischemic tolerance of the myocardium estimated by the LVEDP dynamics during reperfusion**

A particularly significant effect of the action of mAb-TNF-α is the inversion of the negative inotropic effect into a positive inotropic effect at the action of ET-1, which was manifested by increasing the LVSP stimulation value by 8.5%, a fact that was accompanied by the increase CO

at a rate similar to the control group: 14.3% vs 15.7%. The chronotropic response was also similar to the control effect, given the increase in FCC at the respective rates: 4.2 vs 4.6%.

In the context of the coronary benefit of EET-11,12 attested in the group with Dx (i.e., the Vanhoutte phenomenon), it is important the inotropic response of the isolated heart to the action of ET-1 in condition of pre-treatment with epoxyeicosatrienes, as well as the nature of its modification under the action of mAb-TNF-α. As reported above, preconditioning of the isolated heart with ET-1 affects myocardial ischemia-reperfusion tolerance, and preconditioning and postconditioning of the heart with EET-11,12, on the contrary, improved LVEDP and LVSP dynamics during ischemia and reperfusion. Pre-treatment of the working isolated heart perfused with EET-11,12 revealed a valuable benefit of epoxyeicosatrienes, namely the appearance of the positive inotropic response at the peak of stimulation of the heart with ET-1 in the Dx group (figure 10). Thus, the increase in LVSP in the treatment group was 65% higher (14 vs 8.5%), and the increase in CO higher by 32% (21 vs 16%).

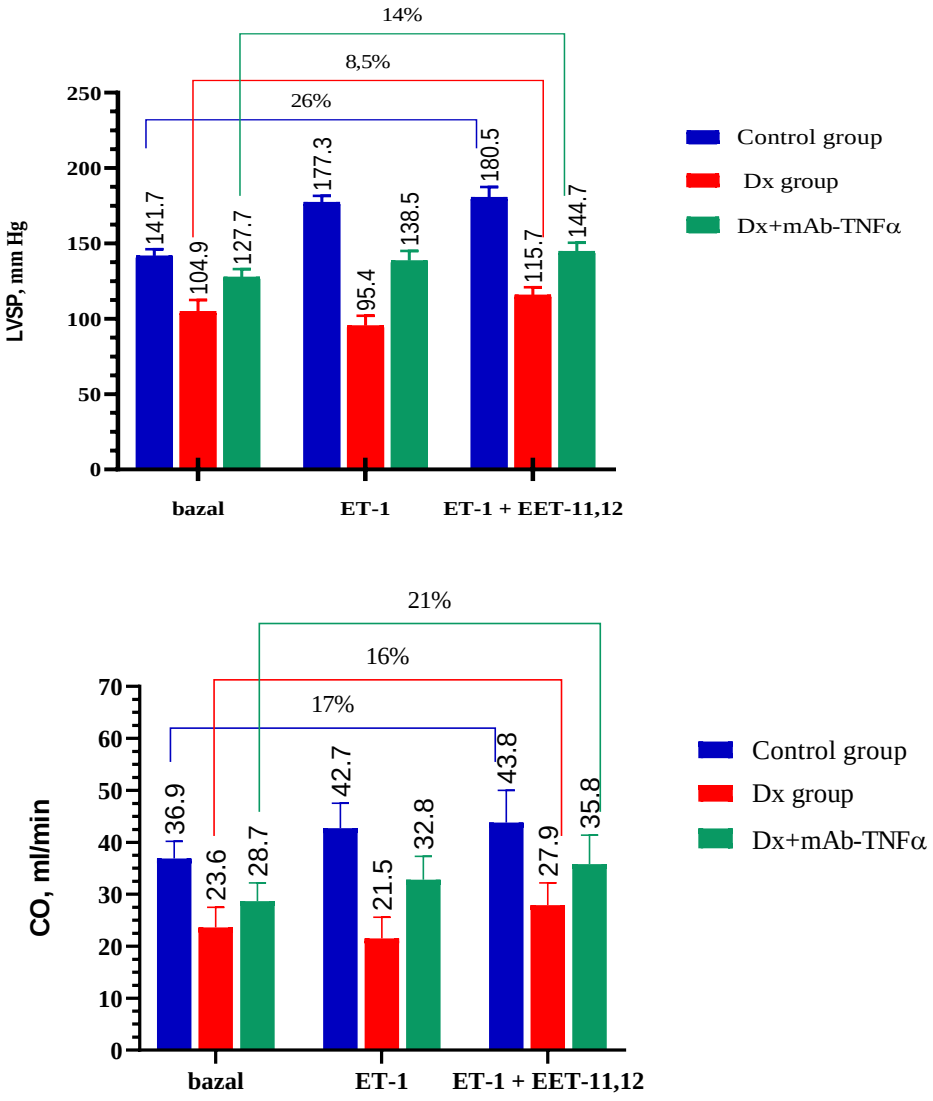

Figure 10. **The changes of LVSP and CO under the stimulation of the heart with ET-1 in condition of EET-11,12 action**

The described phenomenon indicates the role of inflammation in the pathogenesis of a crucial physiopathological pattern of Dx cardiotoxicity, the negative inotropic effect of the heart to the action of ET-1, and on the other hand the biological significance of EET-11,12 translated by their ability to improve myocardial inotropism. In this context, it is conceptually important the connection between the coronary Vanhoutte phenomenon and the influence of EET-11,12 on the inotropic response of the heart in condition of mAb-TNF-α action, or in other words, mAb-TNF-α modulates the Vanhoutte phenomenon. Remarkably, the tolerance of the myocardium to the impact of tachycardia is indispensable for the feasibility of the Vanhoutte phenomenon, and this finding brings new evidence to one of the most common pathological processes of the myocardium: the stunned and hibernating myocardium [11, 12].

Overall, the functional benefits of mAb-TNF-α demonstrated *in vitro* on isolated heart perfusion models (isovolumic and working mode) highlight the role of inflammation in triggering and promoting Dx cardiotoxicity, and on the other hand substantiate and justify the perspective of its mitigation by anti-cytokine treatment. At this point, the benefit of mitigating the effect of TNF-α is also important in the evolution of heart failure of various origins [13, 14], a fact that shows the role of TNF-α contribution in the pathogenesis of cardiac pathology, inclusive the doxorubicin cardiotoxicity.

GENERAL CONCLUSION

1. The cardiotoxicity of doxorubicin estimated in our study on the model of isolated rat heart perfusion excelled by impaired myocardial contractility, diastolic relaxation and left ventricular pump function, so that the significant deviations of the explored functional indices compared to the control level were detected in limits 21.9-168%. Cardiac dysfunction was associated with the impairment of coronary reactivity, manifested by the reduction of CFR to the action of acetylcholine up to 48.9%, the increase in the supply of B1 receptors of bradykinin in promoting the coronary Gregg phenomenon and the preservation of coronary reactivity mediated by the hyperpolarization mechanism imminent to the action of hydrogen peroxide and of epoxyeicosatrienes.
2. The disorders of the regulation of the isolated heart in hemodynamic and neuroendocrine effort is determined in the cardiotoxicity of doxorubicin by the incompetence of the isovolumic relaxation and contraction of the heart, a fact that was characterized by the decline in the value of the indices -dP/dTmax and +dP/dTmax by 25.3-32% in minimal left ventricular filling and maximal aortic cavity resistance. Also, in the pathogenetic aspect, the development of the negative inotropic response of the heart to the action of ET-1 is important, as well as the risk of myocardial tolerance in the impact of ischemia-reperfusion, given the significant increase in end-diastolic pressure by 53% and the decreasing of systolic pressure in the recovery of coronary flow by up to 67%.
3. The attenuation of inflammation by daily i/p administration of the TNF-α antagonist (0.5 mg/kg) during the 2-week period immediately preceding the action of doxorubicin (cumulative dose of 16 mg/kg) conclusively reduced anthracycline cardiotoxicity, given the improvement of the conclusion of the heart's ability to adapt to hemodynamic, neuroendocrine stress and to the action of ischemia-reperfusion, a fact that arguments the contribution of inflammation in the evolution of doxorubicin cardiotoxicity and its pejorative action on the functional reserves of the myocardium.
4. The action of the TNF-α antagonist (mAb-TNF-α) was imposed by significantly increasing the -dP/dTmax value by 27% in the effort with minimal filling of the left atrium and the +dP/dTmax value by 14% in the effort with resistance, which was associated with the augmentation of the pump function of the left ventricle, so that the aortic jet increased by 88% and 34% in the volume effort and respectively in the resistance effort. Improvement in diastole is an obvious functional benefit of mAb-TNF-α action by the optimization of coronary pressure – end-diastolic pressure as well as end-diastolic volume – end-diastolic pressure relationships.
5. An important effect of the action of mAb-TNF-α on the attenuation of doxorubicin cardiotoxicity is the appearance of the positive inotropic response to stimulation of the isolated heart with ET-1 manifested by an increase in systolic pressure and cardiac output by 8.5 and 14.3%, respectively, characteristic for the negative inotropism of the myocardium inherent in the doxorubicin affection without the modulation of inflammation, which was manifested by the decrease in the value of these indices at the peak of the stimulation by an average of 9%.
6. The attenuation of inflammation by TNF-α antagonist increased endothelium-mediated coronary functional reserve, so that the increment of coronary flow under the action of acetylcholine increased by 42%. At the same time, it is important to mention the increase in the rate of coronary flow to the action of bradykinin by 72%, the given effect being based, unlike the doxorubicin disease, on the increase the ratio of B2/B1 receptors in the coronary

dilation of the nonapeptide. This benefit is connecting with the notable increase in myocardial resistance to ischemia and reperfusion.

PRACTICAL RECOMMENDATIONS

1. The results obtained during *in vitro* estimation of the particularities of heart dysfunction *vis-à-vis* the cardiotoxicity of doxorubicin indicate the role of the incompetence of the relaxation and isovolumic contraction phases of the heart in compromising its adaptation to hemodynamic and neuroendocrine effort, so it is recommended:
   A – The use in the Cardiology-Oncology medical field of echocardiographic indices that reflect the feasibility of these phases (e.g., the Tei index, the acceleration time and the deceleration time, as well as the velocity -dP/dTmax and +dP/dTmax) as true predictors of early heart failure, as a manifestation of doxorubicin cardiotoxicity in anthracycline-treated patients.
   B – The involvement in the didactic process of the students of the pathophysiology discipline and of the residents of the Cardiology and Oncology speciality, the conceptual entity aimed the role of inflammation in the initiation and evolution of doxorubicin cardiotoxicity, as well as the functional peculiarities characteristic for the diagnosis and prognosis of heart disease.
2. The approach to design the strategy of primary and secondary prophylaxis of the functional disturbances of the myocardium inherent to the cardiotoxicity of the TNF-α antagonist doxorubicin (i.e., Infliximab) with the aim of attenuating the inflammatory response and, respectively, the dysfunction of the heart, will permit to reach the cumulative curative dose of anthracycline.

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- **Rezumate/abstracte/teze în lucrările conferinţelor ştiinţifice naţionale şi internaţionale**

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- **Participări cu comunicări la forumuri ştiinţifice:**
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