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REACTIVE MORPHOLOGICAL CHANGES OF RATS' HEARTS WITH AN EXPERIMENTAL UNDIFFERENTIATED DYSPLASIA OF CONNECTIVE TISSUE

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Abstract

In modern literature, there are sufficient data concerning clinical manifestations of connective tissue dysplasia, its diagnosis, principles of correction, but works devoted to factors that lead to dysplasia, and morphological manifestations of this condition are insufficient.

The aim: to establish a reactive morphological changes of the heart of rats with an experimental undifferentiated dysplasia of connective tissue.

Materials and methods. As an experimental model of undifferentiated dysplasia of connective tissue it is selected a model of intrafetal antigens injection at the 18 th day of dated pregnancy. The object of the study was 144 hearts of white laboratory rats, which were divided into 3 groups: - The 1st one – intact animals; the 2nd group consisted of experimental

animals; the 3rd group consisted of control rats. Histochemical, histological methods, statistic methods were used in the work.

It is settled that in rats with an experimental undifferentiated dysplasia of connective tissuethe thinning of the hearts' walls from birth up to the 45th day of life is defined compared to control, it is most pronounced at the 1st and 9th day of life. In the group of experimental rats, starting from the 21st day of life up to the end of the observation period the relative area occupied by connective tissue fibers is lower compared to control. This happens mainly due to the lower proportion of collagen fibers type III from the 14th up to the 45th day of life. In experimental rats with undifferentiated dysplasia of connective tissue a significant thinning of the intima-media complex of the arteries of the heart was observed throughout the observation period with the most pronounced changes at the 3rd (3,35±0,09 μm vs 4,95±1.03 μm in controls) and the 21st (7,57±0,25 μm vs. 9.5±of 1.62 μm in controls) days of life, $p<0.01$.

Keywords: undifferentiated dysplasia of connective tissue, heart, collagen fibers, lymphocytes, intrafetal antigen injection

Introduction.

Cardiovascular diseases form one of the urgent problems of modern medicine. According to the WHO (2018), pathology of the cardiovascular system occupies the first place in the structure of mortality from non-infectious causes and composes 44 % [39]. In particular, in Ukraine, which according to this criterion takes one of the first places in the world, this index rises up to 67 % [7, 9].

At the end of the XXth century, the English epidemiologist David Barker, formulated a theory that the condition of fetal development influenced on the formation of health and diseases in future life. This theory received confirmation in numerous experimental studies, where epigenetic changes were determined [3, 4, 5, 20, 24, 27]. Among factors that can affect the fetus during the prenatal period, there may be some drugs that are assigned to women in the third trimester of pregnancy, and infectious agents of bacterial origin, that cross the placental barrier [26].

The role of connective tissue in the functioning of organs and systems of the body is undeniable, because it fulfils many functions, participates in ensuring maintenance of the organism homeostasis, so the metabolism impairment of connective tissue leads to pronounced structural changes of various organs, contributes to the formation of secondary (associate) pathology of the internal organs and systems. dysplasia of connective tissueis a group of genetically heterogeneous and clinically polymorphic pathological conditions characterized by disturbance of formation of connective tissue in the embryonic and postnatal periods, as well as combining a number of genetic syndromes (Marfan, Ehlers-Danlos) and undifferentiated (nonsyndromic) forms with multifactor development frameworks (UDCT). Unlike syndromic forms of connective tissue dysplasia, manifestations of the UDCT is not as pronounced and are often left without proper attention. However, the versatility of the connective tissue defect in UDCT leads to a variety of visceral changes, some of which can have serious clinical consequences. A variety of clinical manifestations of undifferentiated dysplasia of connective tissue (UDCT) complicates its diagnosis [30]. Undifferentiated forms of dysplasia of connective tissue present a genetically heterogeneous group of conditions, which are based on a systematic defect of the connective tissue, result from genetic defects, some medical or biological factors, such as complicated obstetric history (fetal hypoxia, preeclampsia, anemia, and use of drugs), intestinal dysbiosis [14]. In the basis of dysplasia of connective tissueis a violation of collagen synthesis, which manifests itself in the insufficiency or deficiency of fibers development associated with a decrease and/or impaired metabolic activity of fibroblasts [2, 22,30, 32]. Systemic lesions in cases of dysplasia of connective tissueare confirmed by the presence of pathology of the cardiovascular system, musculoskeletal system, various parts of the gastrointestinal tract, including reactive changes in pancreas, primary caries, violation of motor function of the intestine, dyskinesia of bile-excretory tract, the presence of dolicosigma etc. [2, 30].

In modern literature, there are sufficient data concerning clinical manifestations of connective tissue dysplasia, its diagnosis, principles of correction [25, 35, 40], but works devoted to factors that lead to dysplasia, and morphological manifestations of this condition, as well as data reflecting the regularities of morphogenesis of connective tissue in normal and in modeling changes in the system mother-placenta-fetus in the early stages of postnatal ontogenesis are insufficient.

The aim: to establish a reactive morphological changes of the heart of rats with an experimental undifferentiated dysplasia of connective tissue.

Materials and methods. As an experimental model of undifferentiated dysplasia of connective tissue it is selected a model of intrafetal antigens injection at the 18 th day of dated pregnancy [11, 12, 37]. The object of the study was 144 hearts of white laboratory rats, which were divided into 3 groups: - The 1st one – intact animals; the 2nd group consisted of experimental animals, each of which at the 18th day of fetal development was injected by 0.05 ml of antigen in 0.9% NaCl; the 3rd group consisted of control rats, injected by 0.05 ml of 0.9% NaCl at the 18th day of of dated pregnancy. Rats were born full term without developmental malformations and were absolutely healthy. It is said that all animals with any symptoms of a disease were avoided to take at experiment. Sex differences were not considered. The formation of a control group of rats is due to the need to eliminate the influence of surgical intervention in the prenatal period on the obtained changes in the experimental group. A liquid purified Staphylococcal toxoid, dissolved in 10 times (from 10 to 14 units of bind in 1 ml) was chosen as an antigen. The election of the staphylococcal toxoid as antigen was made due to the extensive colonization of genital tract of pregnant women, amniotic fluid and placenta be staphylococci [33]. Animals were contained in standard conditions of vivarium according to "European Convention for the protection of vertebrate animals used for experimental and other scientific purposes" (Strasbourg, 18.03.86 G.) and the Law of Ukraine № 1759-VI (15.12.2009) On the Protection of Animals from Cruelty. Food and water were made available ad libitum. Morphological structure of heart was examined at days 1st, 3rd, 5th, 9th, 14th, 21th, 30th and 45th after birth. Fixation of histological material was carried out in 10% neutral formaldehyde. Histochemical, histological methods, statistic methods were used in the work.

Serial histological sections of 4 μm in thickness after dewaxing were stained with hematoxylin and eosin, orsein-fuksin, Mason-staining, van Gieson's staining, impregnation of samples with argentum carbonate was conducted as well, for lymphocyte detection lectinohistochemical reaction with Peanut agglutinin (PNA) (LectinTest, Lviv) was conducted. All samples were embedded in the balm. Photos of histological samples were conducted using CarlZeiss software (AxioVision 4.8). The relative area occupied by the fibers was estimated in the programme ImageJ with an overlay of masks. Percentage values were obtained as the ratio of the number of pixels that corresponded to the studied structures that are specifically colored, to the total number of pixels in the digital image of the sample, with the data obtained in the area of 9000 μm2. The thikness of arterial wall (intima-media complex) was carried out using CarlZeiss software (AxioVision 4.8).

Analysis of the obtained results was conducted by means of statistical methods with the use of computer license program «Statistica for Windows 13» (StatSoft Inc., № JPZ804I382130ARCN10-J). The statistical significance of the obtained differences of indicators in the comparison groups was evaluated using the Mann-Whitney U test and considered to be significant at $p < 0.05$, that is generally accepted for biological and medical researches. The numerical data of the obtained results are presented as M ± m (arithmetic mean ± standard error of the mean).

Ethnical approval. Supporting and withdrawal of animals from experiment was carried out in accordance with the requirements of the European Commission Directive (86/609/EEC), Law of Ukraine № 1759-VI (15.12.2009) On the Protection of Animals from Cruelty.

Results and discussion. The lack of significant changes in the values of the intact and control groups allows to exclude the influence of surgery on the results of the experimental groups.

In the group of rats with an experimental undifferentiated dysplasia of connective tissue the thickness of the walls of the heart changes. Morphometric indices of heart walls thickness in rats with an experimental undifferentiated dysplasia of connective tissue remain lower during the first three weeks after birth, then catch up with the values of control animals, and at forty-five day of postnatal life significantly exceed them (Table 1). Such changes indicate uneven growth of the heart wall in the postnatal period and ilustrate a manifestation of a violation of the normal morphogenesis of the heart on the background of experimental undifferentiated connective tissue dysplasia. Simultaneously the periods of myocardial stratification of the ventricles of the heart also change: a clear visualization of the layers of the myocardium in experimental rats is observed on the thirtieth day of postnatal life, in contrast to control animals, where the morphology of the layers is observed on the twenty-first day of life. Yu. G. Reznichenko (2013) also observed a similar disproportionate development of the heart of rats in the period from the seventh to the sixtieth day after birth in an experiment with intrauterine exposure to antigens of different nature [28]. According to some authors, disorientation and hypertrophy of cardiomyocytes, accompanied by the increased thickness of the hearts' walls, may be a manifestation of hypertrophic cardiomyopathy, associated with the development of undifferentiated connective tissue dysplasia. [16, 34].

Previously, it was also found that in the myocardium of rats after intrafetal injection of antigens the area and number of nuclei of the cardiomyocytes, their nuclear-cytoplasmic ratio had been decreased [10].

During the first two weeks of life in the myocardium of ventricles of rats with experimental undifferentiated dysplasia of connective tissue, the relative area occupied by elastic fibers demostrates a tendency to diminishing in comparison with control group. Later on at the 45th day after birth this index surpasses the data, obtained in control group, however, the described differences do not acquire authentic significance (table 2).

In the ventricular myocardium of rats with experimental undifferentiated dysplasia of connective tissue, the relative area, occupied by collagen fibers in general increases in 1.6 times as in control up to the fifth day of life. Later on up to the 45th day after birth it increases only by 28.4 percent (table 2). Starting from the 21st day of postnatal life the content of collagen fibers in general in the myocardium of experimental rats was significantly lower compared to the control group (Fig.1). However, the analysis of the relative area occupied by collagen fibers type III shows an uneven dynamics of the distribution of these fibers in the ventricular myocardium and significantly lower values starting from the fourteenth day after birth compared to the control rats (Fig.2). Obtained data indicate disproportionate development of cardiac fibrous skeleton. Earlier on the basis of the same model on the example of the knee joint it was set the slowdown of fibers development, changes in the ratio of fibers and extracellular matrix with the prevalence of the latter, the thinning of the collagen fibers of the joint capsule, the early appearance of the elastic fibers in the transitional zone of the joint capsule compared with control, changes of lymphocyte-fibroblast index in the transitional zone of the joint capsule. On the background of obtained data the model of intrafetal antigen injection was proposed as the model of experimental undifferentiated dysplasia of connective tissue [12, 37]. Indeed, among the causes of the development of connective tissue dysplasia, a number of authors identify the pathological course of pregnancy with extragenital pathology of viral and bacterial origin [8, 31]. Dysplasia of connective tissue of the heart appears the so-called minor heart anomalies, which include mitral valve prolapse, false tendon strings, increased trabecularity of ventricles of the heart and dysfunction of the cardiac conduction system [15, 19]. According to the literature, morphological basis of pathological changes during connective tissue dysplasia, is formed by the defects in the structure of connective tissue, including impaired synthesis of collagen type III due to genetic defects and the formation of antibodies to collagen and,

consequently, decreased content of collagen type III in the myocardium of the heart, and, consequently, all collagens as well [18, 19, 25]. According to B. A. Kuznetsov et al. (2018) it is the exact lower content of collagen, that may result in cardiac arrhythmias due to abnormal conduction of excitation [16]. Nowadays it is settled that in adulthood the presence of dysplasia of connective tissueincreases the severity of the clinical course of concomitant diseases and acts as an additional risk factor of coronary artery disease, and systolic and diastolic dysfunction of the ventricles of the heart [6, 29].

Dynamics of the relative area occupied by arterial vessels in ventricular myocardium of rats after intrafetal injection of staphylococcal toxoid has a wavelike nature with a peak result at the 14th day, unlike the control group where it falls at the 30th day of life, and at this observation period, differences between values of these groups gain statistical significance.

The thickness of the intima-media complex of the arteries in the heart of rats with experimental undifferentiated dysplasia of connective tissue had similar dynamics with changes in the index in the control animals, but values were significantly lower during the whole observation period. In particular, at the 21st day after birth the most pronounced changes in the thickness of the walls of the arteries in the experimental group was observed: it was by 19.44 % less than in the control group of animals (table. 3). The most expressed changes took place in the internal elastic membrane and in the middle membrane of the arteries (Fig. 3). The obtained results coincide with the data of the study T. Mitchell (2019) who observed the violation of angiogenesis in the heart of the primates in experimental models of preterm birth associated with infection [20]. The described changes of the heart vessels can increase the risk of coronary heart disease in adulthood [20, 23].

The majority of lymphocytes in the heart represent a part of vascular-associated lymphoid tissue (VALT) and are located mainly in the middle membrane of arterial vessels and periarterially, more often in clusters of 3-4 cells [21]. According to the concept of "Lymphocyte - a factor of morphogenesis", formed by M.A. Voloshyn (2005), the cells of the immune system play a key role during intranatal development of tissues and organs of the fetus [38]. Among these cells, one defines γδ + T-lymphocytes, which on their surface express terminal residues of D-galactose, with which peanut lectin - Peanut agglutinin (PNA) is able to specifically bind [1]. The highest content of PNA $^{+}$ lymphocytes in the ventricular myocardium of rats during the first week after birth coincides with a period of intense morphological changes in the heart, which suggests the participation of PNA^{+} lymphocytes in the normal morphogenesis of rat heart in the early postnatal period.

Dynamics of the content of PNA^+ lymphocytes in the outer layer the ventricular myocardium of heart of rats with experimental dysplasia of connective tissueis of wavy character, with a gradual increase of their amount from birth up to the 9th day of life (1.77 times) and a subsequent decrease up to the 45-th day of life (table. 4), and the content of PNA^+ lymphocytes in subepicardial layer of myocardium of experimental animals at the ninth day of life significantly higher than in control animals (Fig. 4) and exceeds control data in 2.28 times. The content of PNA^+ lymphocytes in the subendocardial layer of animals with an experimental connective tissue dysplasia, was significantly higher in comparison with the control rats, at the 5th, 14th and 21st days of life. The obtained data support the concept of M. A. Voloshyn (2005) that the penetration of fetal antigens of different nature to the fetus leads to premature exit of lymphocytes (including $\gamma\delta^+$ -lymphocytes) from the thymus, that affect the processes of proliferation, differentiation and maturation of cells, thereby changing the pace and timing of the morphogenesis of internal organs [36, 38].

The similar results were obtained in the study of intrafetal effects of antigens injection on the morphogenesis of other organs of rats [11, 13, 17]. Thus, after intrafetal injection of staphylococcal toxoid, the content of PNA + lymphocytes in the ventricular myocardium of rats increases, this may change the process of normal morphogenesis and reduce the adaptive capacity of the heart in the future (Fig. 5).

Thus, in rats with an experimental undifferentiated connective tissue dysplasia, which was modeled by intrafetal antigen injections at the 18 th day of dated pregnancy, there is a mass exit of lymphocytes from the thymus (including $\gamma\delta^+$ PNA+lymphocytes, which fulfil a morphogenetic function) to the periphery, including lymphoid tissue, associated with blood vessels (VALT), which applies to the heart tissue as well. This leads to functional changes of cardiomyocytes and fibroblasts, and is reflected by the reduction of nuclear-cytoplasmic ratio of cardiomyocytes, thinning of the cardiac walls, a decrease in the content of collagen and elastic fibers that form connective tissue skeleton of the heart, and in turn provide elastic properties of the organ, thinning of the arterial walls and decrease of the relative area occupied by the arteries. Further, all this may lead to disorders of myocardial blood supply and violation of its contractile capabilities.

Conclusions

1. In rats with an experimental undifferentiated dysplasia of connective tissue the thinning of the walls of the right and left ventricles and the interventricular septum from birth up to the 45th day of life is settled compared to control, it is most pronounced at the 1st and 9th day of life.

2. In the group of rats with an experimental undifferentiated connective tissue dysplasia, starting with the 21st day of life up to the end of the observation period the relative area occupied by connective tissue fibers is lower compared to the control animals. This happens mainly due to the lower proportion of collagen fibers type III from the 14^{th} up to the 45th day of life. The most pronounced changes are determined at the 21st day (3,74±0,14 % in the experimental group and 5.57±0,38 % in the control), $p<0.01$.

3. Significant thinning of the intima-media complex of the arteries of the heart of rats with an experimental undifferentiated dysplasia of connective tissue was observed throughout the observation period with the most pronounced changes at the 3rd (3,35±0,09 µm vs 4,95±1.03 ám in controls) and the 21st (7,57±0,25 µm vs. 9.5±of 1.62 µm in controls) days of life, $p<0.01$.

Prospects for further research. In the future, it will be carried out an immunohistochemical study of the distribution of different collagen types in the myocardium and in the heart valves of rats with an experimental undifferentiated connective tissue dysplasia.

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2. **Conflicts of Interest:** authors have no conflict of interest to declare.

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Table 1 – Thickness of the hearts' walls of rats (M±m, mcm)

Day	group	Thickness, mcm		
		Left ventricle	Right ventricle	Interventricular septum
1	I	481,78±5,25	218,83±7,21	349,82±6,23
	II	416,31±3,50*	205,16±4,43*	306,17±6,06*
	III	486,44±7,62	221,34±4,59	358,95±4,44
3	I	509,44±4,37	282,92±7,08	387,59±5,82
	II	483,42±11,64	284,09±4,13	377,92±6,52
	III	506,90±9,75	287,15±3,97	384,89±4,16
5	I	624,50±5,99	336,10±5,38	581,92±8,44
	II	627,93±3,28	289,43±2,56*	511,43±5,35*
	III	631,03±6,07	338,05±8,15	596,63±6,17
9	I	761,48±5,12	356,98±2,61	636,20±3,46
	II	641,98±7,88*	290,13±5,23*	560,39±6,69*
	III	763,50±6,07	352,31±7,41	641,89±4,81
14	I	776,62±9,52	357,64±4,09	693,04±9,27
	II	663,40±9,87*	292,02±8,75*	611,13±4,78*
	III	770,41±7,86	356,26±11,39	687,04±5,21
21	I	782,82±4,85	369,33±5,35	728,36±8,92
	II	726,16±13,41*	377,17±7,03	708,81±5,80
	III	777,63±4,55	363,47±4,98	719,46±3,99
30	I	862,90±5,00	358,94±8,45	782,73±6,06
	II	815,88±8,55*	387,98±4,71	758,30±2,11
	III	857,28±8,24	364,45±9,03	771,37±5,65
45	I	899,27±5,68	372,75±15,34	872,96±10,45
	II	923,08±4,93*	405,97±4,15*	901,79±4,22*

	III	890,87±9,71	369,24±4,34	866,07±9,42

Note: I - group of intact rats; II - group of rats with the experimental undifferentiated connective tissue dysplasia; III - group of control rats; * - significant difference compared to control animals, p <0,05.

Table 2 – Content of connective tissue fibres in the myocardium of rats, (M±m, %)

Day of life	Fibers	Relative area, occupied by fibers, %		
		I	II	III
1-st	Collagen	2,77±0,48	2,30±0,27	2,69±0,25
	Elastic	0,58±0,15	0,51±0,28	0,55±0,12
3-rd	Collagen	3,46±0,46	2,74±0,31	3,24±0,26
	Elastic	0,67±0,20	0,65±0,07	0,65±0,11
5-th	Collagen	3,73±0,39	3,82±0,33	3,74±0,39
	Elastic	0,79±0,10	0,75±0,07	0,70±0,08
9-th	Collagen	4,31±0,35	3,83±0,46	4,36±0,32
	Elastic	1,04±0,11	0,82±0,07	0,81±0,09
14-th	Collagen	5,40±0,46	4,04±0,37	5,17±0,42
	Elastic	1,14±0,14	1,27±0,14	1,00±0,11
21-th	Collagen	5,90±0,40	4,33±0,37*	5,69±0,43
	Elastic	1,22±0,28	1,42±0,15	1,03±0,13
30-th	Collagen	6,82±0,75	4,84±0,39*	6,80±0,90
	Elastic	1,39±0,27	1,64±0,15	1,44±0,19
45-th	Collagen	8,07±0,75	5,34±0,47*	7,88±0,34
	Elastic	1,78±0,29	1,88±0,21	1,73±0,28

Note: I - group of intact rats; II - group of rats with the experimental undifferentiated connective tissue dysplasia; III - group of control rats; * - significant difference compared to control animals, p <0,05.

Table 3 – Thickness of the intima-media (M±m, μm) in arteries of the myocardium of rats

group / day	I	II	III
1-st	3,31±0,51	2,80±0,08*	3,46±0,69
3-rd	4,84±0,82	3,35±0,09*	4,95±1,03
5-th	5,51±1,41	4,07±0,10*	5,66±0,98
9-th	6,52±1,08	5,63±0,14*	6,27±1,21
14-th	7,50±1,14	6,18±0,14*	7,13±1,49
21-th	9,31±1,57	7,57±0,25*	9,51±1,62
30-th	9,71±1,80	8,49±0,16*	9,96±1,37
45-th	9,78±1,56	8,15±0,16*	9,94±1,62

Note: I - group of intact rats; II - group of rats with the experimental undifferentiated connective tissue dysplasia; III - group of control rats; * - significant difference compared to control animals, p <0,05.

Table 4 – Number of PNA^+ lymphocytes (M±m) in mm^2 of the myocardium of the ventricles of intact and experimental rats

Day of life	Localization	Group		
		I	II	III
1st	subepicardial	7,7±3,7	16,9±5,5	10,3±4,6
	subendocardial	14,5±4,8	23,7±6,3	14,2±4,8
3rd	subepicardial	7,5±3,5	16,5±5,1	11,3±4,3
	subendocardial	13,7±4,6	22,9±6,3	14,3±5,4
5th	subepicardial	14,0±4,6	20,5±5,5	15,4±5,4
	subendocardial	20,5±5,5	34,2±6,8*	17,3±5,1
9th	subepicardial	10,0±3,9	30,5±6,5*	13,1±4,8
	subendocardial	15,1±4,8	23,2±5,9	17,7±5,2
14th	subepicardial	10,7±4,2	20,7±5,5	12,5±4,5
	subendocardial	12,6±4,2	22,0±5,9*	7,8±3,7

21st	subepicardial	5,4±2,9	12,3±4,4	8,9±3,9
	subendocardial	9,4±4,0	16,7±4,9*	5,1±2,9
30th	subepicardial	6,2±3,4	8,0±3,9	5,6±3,1
	subendocardial	5,9±3,2	12,0±4,3	6,1±3,4
45th	subepicardial	3,0±2,0	5,9±3,2	3,2±2,2
	subendocardial	2,7±1,9	5,7±3,2	3,2±2,2

Note I - group of intact rats; II - group of rats with the experimental undifferentiated connective tissue dysplasia; III - group of control rats; * - significant difference compared to control animals, p <0,05.

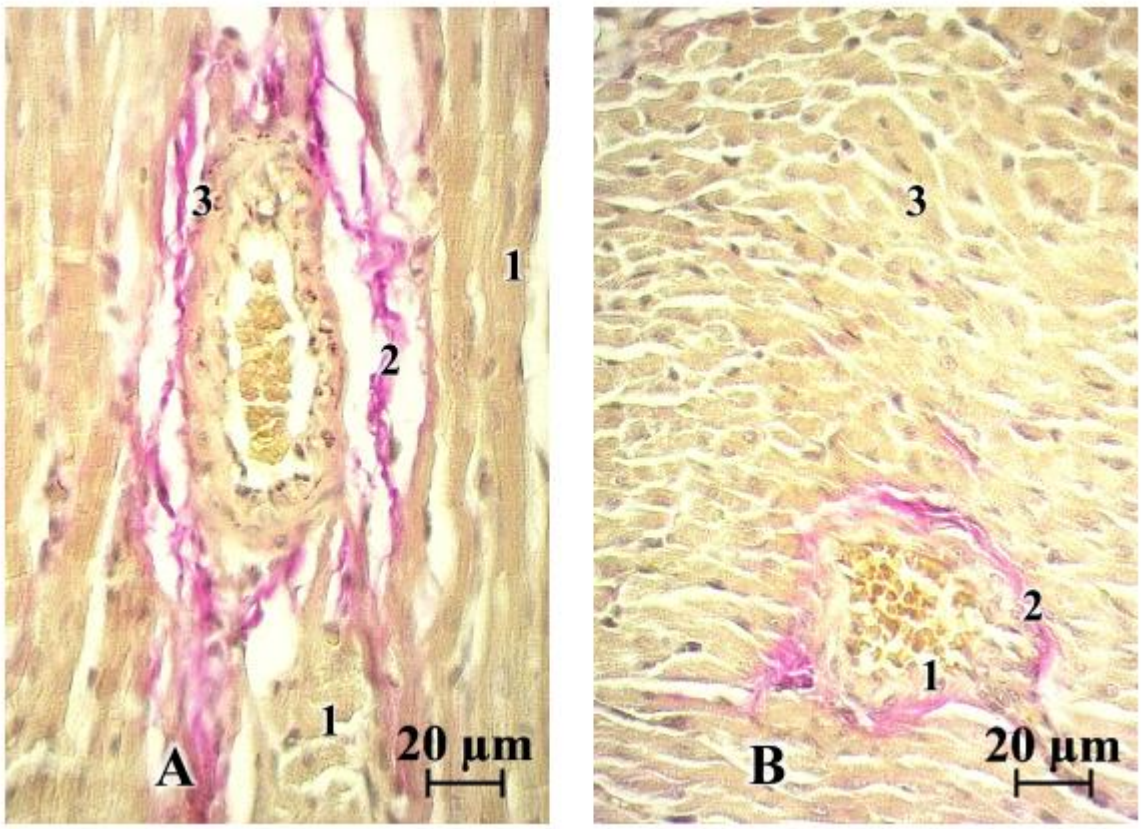

Figure 1 – Miocardium of the left ventricle of rats at the 21-st day of life. Van Gieson's staining. A – control rat; B – rats with experimental connective tissue dysplasia. 1 – collagen fibres; 2 – arterial vessel; 3 - myocardium.

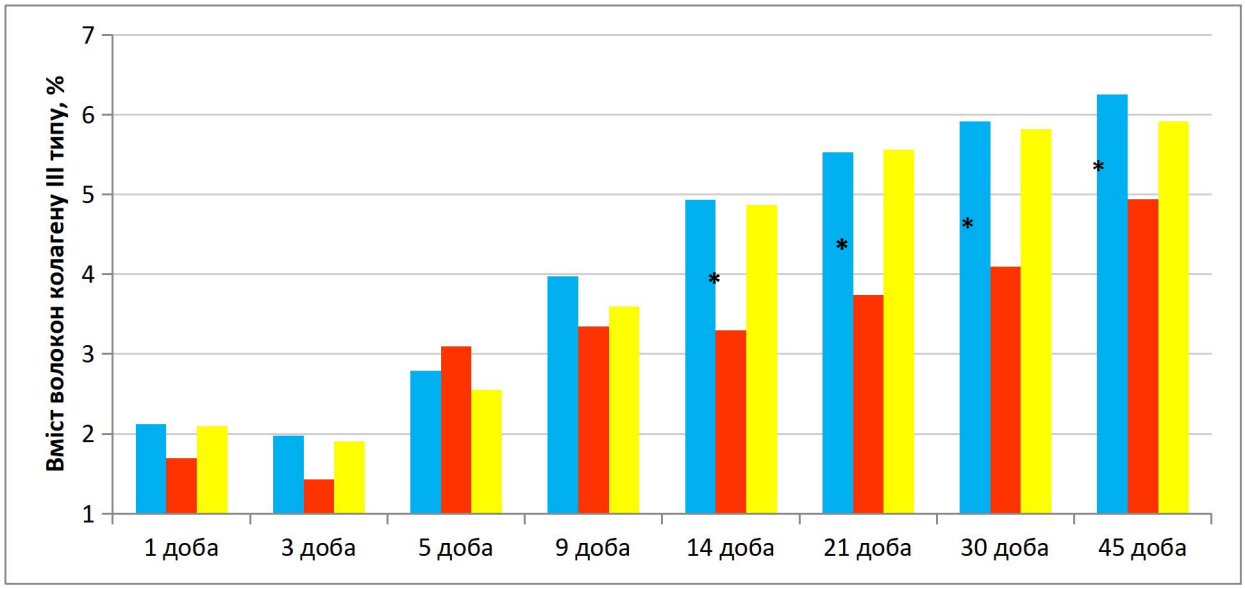

Figure 2 – Dynamics of the relative area ,occupied by fibers of collagen type III in left ventricle myocardium of rats.

Notes: blue – intact rats, red rats after votron the introduction of staphylococcal toxoid, yellow control rats. * – significant difference compared with the control animals, p<0.05.

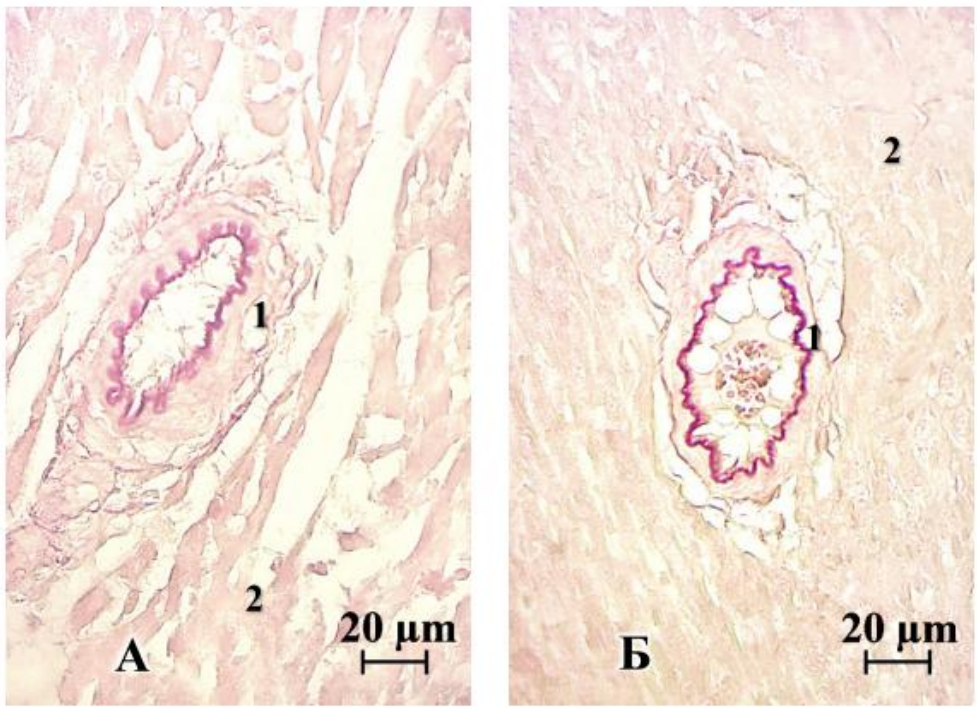

Figure 3 - Arteries in the myocardium of the left ventricle of rats at the 45th day of life. Staining: orcein-fuksin. A - control rats; B - rats with experimental undifferentiated connective tissue dysplasia. 1 - arterial vessel; 2 - myocardium.

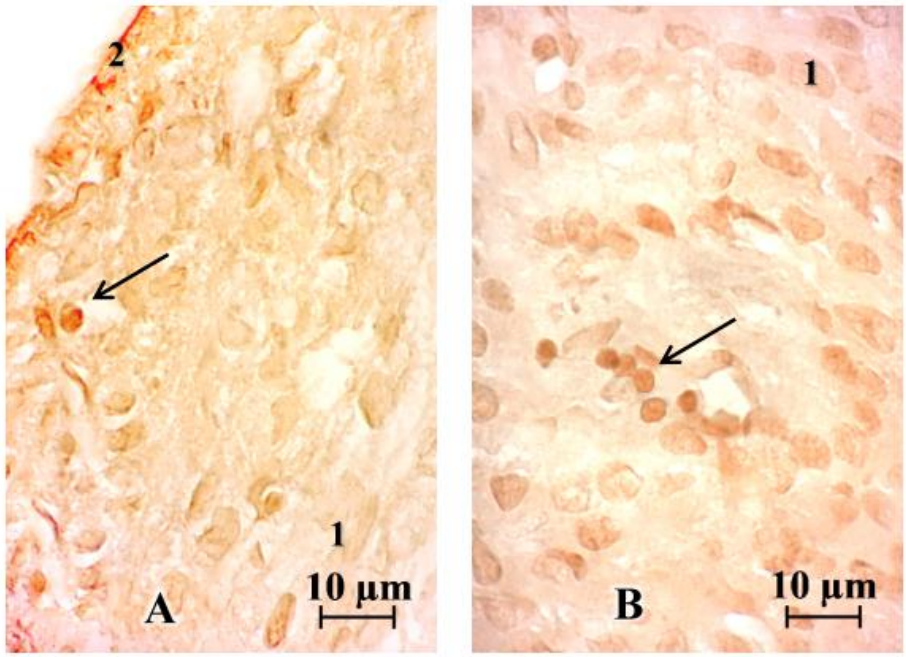

Figure 4 – Location of the PNA+lymphocytes (arrow) in subepicardial layer of the left ventricular myocardium of rats at the ninth day of life. A - control rats; B - rats with an experimental undifferentiated dysplasia of connective tissue. 1 – nucleus of cardiomiocyte, 2 – the epicardium.

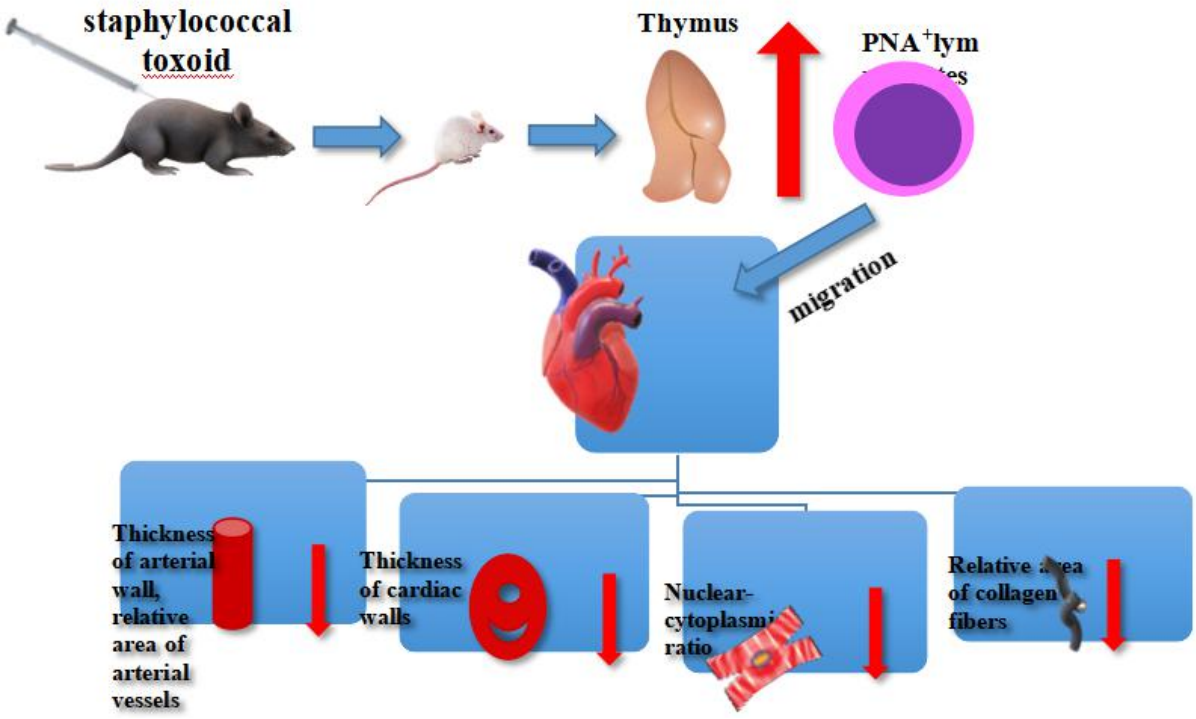

Figure 5 – Diagram of the morphological changes of the myocardium after prenatal exposure to staphylococcal toxoid.

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