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Regenerative Reactions of the Myocardium in Plastic Insufficiency of Cardiomyocytes during Ontogeny

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Structural reorganization of the myocardium was studied in rats of various age groups (periods of progressive and regressive growth) with anthracycline-induced regenerative and plastic insufficiency. The specific features included the prevalence of cardiomyocyte lysis, diffuse and microfocal changes, and diffuse or microfocal cardiosclerosis. During the late ontogeny, myocardial damage was characterized by more pronounced cardiosclerosis (primarily microfocal cardiosclerosis). The development of regenerative and plastic insufficiency of cardiomyocytes determines dilatation remodeling of the heart in rats of various age groups. Comparative study of the morphogenesis of heart failure in rats of various age groups showed that cardiotoxic exposure during the early ontogeny induced more pronounced remodeling of the heart compared to that in late ontogeny. Differences in proliferative activity of cardiomyocytes and ability for hypertrophic growth are the main cellular mechanisms of age-related features of structural reorganization.

Key Words: *myocardium; ontogenetic periods; doxorubicin; morphometry*

Evaluation of structural and molecular mechanisms of regenerative and plastic insufficiency of the myocardium in various periods of ontogeny, *i.e.*, molecular and biological changes in cardiomyocytes (CMC) is required for understanding of the main principles of cell protection and functional compensation. Anthracycline-induced cardiomyopathy is a convenient model for studies of the development of regenerative and plastic insufficiency of the heart [3,4,6]. This model allows us to evaluate a variety of structural and functional changes in CMC and stromal cells, degree of cell death, and specific features of regeneration. Understanding of these mechanisms is important for the development of optimal chemotherapeutic regimens and prevention of side effects (particularly in cancer children) [9,15].

The exposure of myocardial cells to cytostatic and cytotoxic drugs is followed by not only cell injury and death [1,2], but also regenerative response. The nature and degree of this reaction depend not only on the type of damage, but also on the age-related characteristics of reparative processes [14]. No systemic studies of structural reorganization in the myocardium and evaluation of regenerative properties under conditions of plastic insufficiency were performed.

This work was designed to study structural reorganization of the myocardium in rats of various age groups with anthracycline-induced regenerative and plastic insufficiency of the heart.

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MATERIALS AND METHODS

Complex morphological study of the myocardium in male Wistar rats was performed during various periods of progressive (1 month) and regressive growth (23 months). Groups 1 and 2 consisted of 1-month-old animals (n=40) and 23-month-old animals (n=18), respectively. Doxorubicin hydrochloride (Ferane) in a single dose of 5 mg/kg was injected intraperitoneally for modeling anthracycline-induced cardiomyopathy. The samples were taken 5, 14, and 21 days after treatment. Control rats of the same age (3 animals per group) received an intraperitoneal injection of physiological saline (equivalent volume). Before and during the study, all animals were kept in a vivarium under standard conditions, fed complete diet, and had free access to water.

Myocardial samples were fixed in 10% neutral formalin and embedded into paraffin. The sections were stained with hematoxylin and eosin (van Gieson technique). PAS reaction was conducted. Myocardial samples were fixed in 4% paraformaldehyde, postfixed in 1% osmium tetroxide, and treated by the standard method to obtain semithin sections. Semithin sections were stained with 1% azure II solution and subjected to morphological and stereological analysis. The examination was performed under a Leica DM 4000B microscope.

Stereological analysis was performed routinely [5]. The volume and surface density of the major structural components was evaluated in myocardial tissue. The secondary parameters were evaluated from the primary stereological parameters. These parameters illustrate a quantitative relationship between various components of the stroma and parenchyma. The diameter of CMC was calculated by means of Leica QWinV3 software. Alkaline dissociation of fixed tissues was used to assay the total population of CMC in the heart [6].

The mean values, dispersion, and errors of means were calculated. The significance of differences was evaluated by Student's t test.

RESULTS

Injection of doxorubicin hydrochloride in a single dose of 5 mg/kg did cause the death of animals from both age groups. Despite the development of dyspepsia in young rats, they were characterized by a progressive increase in body weight: it increased by 31 (p<0.01) and 47% (p<0.01) on days 14 and 21, respectively. These changes were probably associated with progressive ascites (Table 1). The weight of the heart increased progressively and on days 14 and 21 this parameter surpassed the control level by 26 (p<0.05) and 44% (p<0.05), respectively. In old rats body weight increased on days 5 (by 14%; Table 1). The weight of the heart in most animals was reduced after 5 and 21 days (by 18 and 15%, respectively). Cardiac remodeling (dilation) was found in young and old animals.

The total number and diameter of CMC were measured to evaluate the cellular mechanisms of cardiac remodeling during anthracycline-induced cardiomyopathy. The concentration of CMC per 1 mg heart tissue from 1-month-old rats decreased significantly (by 24%) on day 14 after single injection of doxorubicin (5 mg/kg) and remained unchanged in the follow-up period. However, these changes were not accompanied by a significant decrease in the total number of CMC. It was related to the maintenance of cell proliferation in young animals. It should be emphasized that the diameter of CMC in young animals decreased insignificantly on day 5 after treatment (by 8%). Hypertrophy of CMC was revealed in the later period (21st day). The diameter of cells increased by 8%, which contributed to a significant increase in the weight of the heart in young animals (by 37%; Table 1).

The concentration of CMC in 23-month-old animals decreased after 14 days (by 12%; Table 1), but increased by the 21st day (by 13%). The total number of CMC in the heart of old animals was reduced by 27% on day 5. These changes were probably associated with a decrease in the weight of the heart (by 18%). On day 14 the total number of CMC in the heart remained below the control (by 19%), which was related to a decrease in the concentration of cells and weight of the heart. Despite a slight increase in the concentration of cells, the total number of CMC in the heart remained low on day 21 (by 14%). A decrease in the total number of CMC in old animals was associated with an imbalance between cell death and proliferation. However, no significant changes in the diameter of CMC were found in these rats. This parameter was decreased by 12% on days 5 and 14 after drug treatment, but returned to normal in the follow-up period.

Different changes in the concentration and number of CMC in the heart of young and old animals after single treatment with doxorubicin are associated with variations in the regenerative ability of CMC. The maintenance of proliferative activity of CMC and ability for the hypertrophic growth in young animals provide the recovery and increase in the weight of the heart. Severe hypertrophy of CMC and heart in young animals may be considered as a side effect of doxorubicin during early ontogeny.

The rats of both age groups were characterized by similar changes in myocardial tissue after single injection of doxorubicin. A mosaic staining pattern of CMC with acid dyes was observed on day 5. It was related to the presence of CMC with normal tinctorial properties, profound lysis of sarcoplasm, and contraction of CMC

TABLE 1. Body Weight, Weight of the Heart, and Quantitative Analysis of CMC Population in the Heart of Wistar Rats after Injection of Doxorubicin in a Single Dose of 5 mg/kg (*M*±*m*)

Parameter		Control	Time after doxorubicin injection, days		
			5	14	21
1-month-old rats	body weight, g	108.5±1.5	114.0±4.9	142.6±1.2**	159.5±3.7**
	weight of the heart, g	0.467±0.015	0.488±0.036	0.590±0.031*	0.670±0.057*
	relative weight of the heart, mg/g	4.31±0.14	4.27±0.15	4.14±0.25	4.19±0.27
	CMC diameter, μ	13.87±0.48	12.80±0.74	14.07±0.64	15.03±0.50
	concentration of CMC per mg tissue, $\times 10^3$	7.10±0.79	8.00±0.61	5.40±0.18	5.40±0.36
	absolute number of CMC in the heart, $\times 10^3$	3250.0±432.0	3639.0±471.3	2943.7±99.2	3307.3±430.3
23-month-old rats	body weight, g	666.3±31.8	573.3±71.0	672.0±100.9	676.3±130.0
	weight of the heart, g	2.07±0.14	1.70±0.05	1.97±0.03	1.76±0.10
	relative weight of the heart, mg/g	3.11±0.17	3.10±0.51	3.03±0.36	2.72±0.32
	CMC diameter, μ	21.56±1.15	19.00±0.27	19.12±0.34	22.23±0.52
	concentration of CMC per mg tissue, $\times 10^3$	12.70±0.96	12.20±0.94	11.20±0.28	14.40±0.78
	absolute number of CMC in the heart, $\times 10^3$	14,860.0±843.0	10,783.0±632.0*	12,055.0±507.0*	12,822.0±1146.0

Note. Here and in Tables 2 and 3: *$p<0.05$ and **$p<0.01$ compared to the control.

in the myocardium. These cells exhibited a nonspecific PAS-positive staining of the sarcoplasm (not associated with glycogen). These changes persisted during various periods of the study. Many nuclei of CMC in young and old animals were translocated to the subsarcolemmal region (Fig. 1, *a*). Sarcoplasmic lysis was observed in 50% CMC of young and old animals. Muscle fibers were sometimes broken by the intercalated discs. It should be emphasized that lytic changes were more pronounced in CMC of old animals (Fig. 1, *d*). These differences were probably related to the deceleration of recovery processes [10].

Hemodynamic changes manifested in the plethora of subepicardial veins and capillaries (Fig. 1, *a*) and artery spasm. Hemodynamic disturbances were followed by the development of interstitial edema and lymphostasis. Diffuse mononuclear infiltration of the stroma was found in all layers of the myocardium. Numerous fat cells were characterized by perivascular location. Moderate perivascular and interstitial sclerosis was revealed in the myocardium of old animals.

Pathomorphological changes in the myocardium after treatment with doxorubicin are typical of various drugs of the anthracycline family. They constitute the morphological basis of anthracycline-induced cardiomyopathy [8,11,12]. A specific feature of the effect of anthracycline antibiotics is damage to a considerable number of intracellular structures in CMC, which contributes to the development of insufficiency [7,8,13].

Mosaic staining pattern of CMC was observed in rats of both age groups on day 14 after treatment. In young animals, the percentage of CMC with lytic changes did not exceed 25% of the total cell number. Focal lysis of the CMC sarcoplasm was more often found in old animals than in young specimens (particularly in the perinuclear region; Fig. 1, *e*). The nuclei of some CMC were displaced to the subsarcolemmal region. CMC were characterized by the phenotypic heterogeneity. In addition to hypertrophic cells, the thinned and dystrophic cells were revealed within the same muscle fiber.

Severe edema of the myocardial stroma was accompanied by strong separation of the muscle bindles and fibers. Hemodynamic disturbances were shown to persist during this period. It was manifested in venous and capillary plethora, strong dilation of lymphatic capillaries (Fig. 1, *b*), and presence of hemorrhagic focuses. The arterial adventitia was loosened and infiltrated by mononuclear cells. The focuses of stromal infiltration were small in young animals. They were

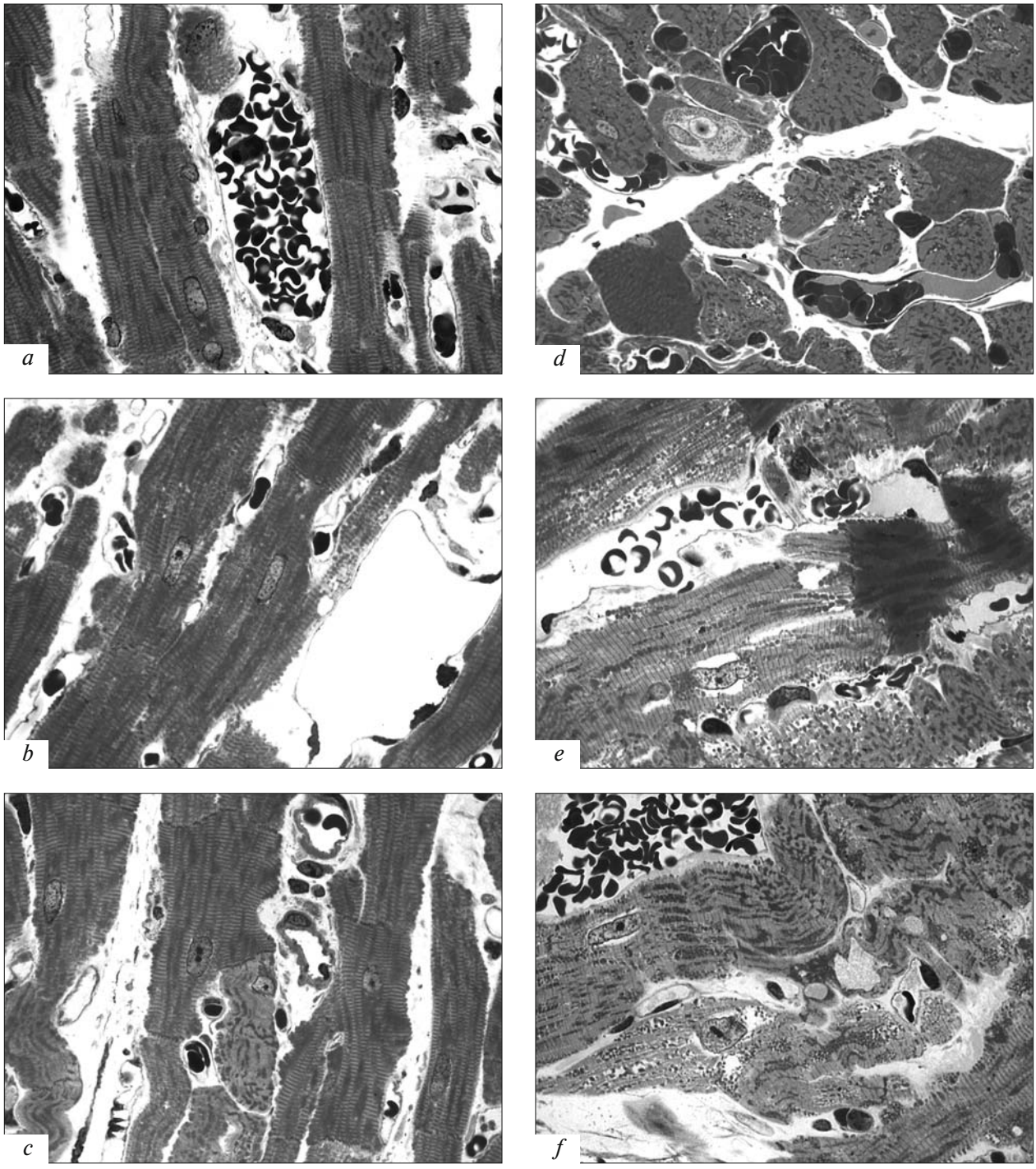

Fig. 1. Myocardium of 1-month-old (*a-c*) and 23-month-old Wistar rats (*d-f*) after single treatment with doxorubicin. Semithin sections. Azure II staining, ×1000. (*a*) Severe venous and capillary plethora, translocation of CMC nuclei to the subsarcolemmal region on day 5; (*b*) capillary plethora and lymphostasis on day 14; (*c*) collagenation of the myocardial stroma on day 21; (*d*) strong lysis and contraction of CMC, vascular plethora on day 5; (*e*) "depletion" of the perinuclear region and strong lysis of the sarcoplasmic matrix on day 14; (*f*) partial necrosis and strong lysis of CMC on day 21 after doxorubicin injection.

mainly found in the perivascular region and sites of CMC atrophy or death. Microfocal cardiosclerosis was sometimes found in the myocardium of old animals. Numerous mast cells were located in the perivascular and interstitial space.

Loosening of the myocardium on day 21 was associated with severe perivascular, intermuscular, and interfibrillar edema of the stroma (particularly in the middle layer). CMC were characterized by a mosaic staining pattern with acid dyes. Focal and diffuse lysis

of the sarcoplasm was found in CMC. The degree of lysis was high in some cells. The ratio of abnormal CMC was above 50% of the total cell number. CMC with focal lysis and partial necrosis of the sarcoplasm were often found in the myocardium of old animals (Fig. 1, *f*). Muscle fibers were broken by the intercalated discs. Focal mononuclear infiltration in the site of CMC death was often revealed in the myocardium of these rats. The observed changes illustrate myocardial decomposition. Severe hemodynamic disturbances and interstitial edema were present during this period. Focal mononuclear infiltration and diffuse cardiosclerosis of the myocardium were typical of old animals. Young animals were characterized by moderate collagenation of the stroma (Fig. 1, *c*).

Spatial reorganization of the myocardium in rats of both groups was revealed during various periods after doxorubicin injection. It was manifested in a decrease in the surface density and surface/volume ratio of CMC (Tables 2 and 3). The surface/volume ratio of CMC nuclei was also reduced under these conditions. A decrease in the volume and surface density of capillaries (by 12-28 and 15-25%, respectively), endothelial cells (by 24-46 and 11-44%, respectively), and connective tissue cells (by 6-42 and 30-40%, respectively; except for the 21st day) was observed in the myocardium of young animals. We revealed a decrease in the surface/volume ratio of capillaries-to-CMC (by 14-26%) and volume ratio of capillaries-to-CMC (by 11-30%).

TABLE 2. Stereological Analysis of the Myocardium in Wistar Rats after Injection of Doxorubicin in a Single Dose of 5 mg/kg (*M*±*m*)

Parameter			Control	Time after doxorubicin injection, days		
				5	14	21
1-month-old rats	volume density, mm^3/cm^3	CMC	798.7±13.8	794.6±30.5	812.4±17.5	768.9±4.1
		CMC nuclei	13.0±2.8	14.4±5.0	12.7±0.3	10.6±0.9
		capillaries	70.0±9.5	61.7±6.8	50.4±3.4	58.0±5.1
		endothelial cells	9.1±1.8	6.9±1.0	4.9±1.2	5.7±1.4
		connective tissue cells	10.9±3.1	6.3±0.8	10.2±3.7	10.4±2.3
		fibers and basic substance of the connective tissue	111.3±12.9	130.4±22.2	122.1±21.7	138.3±15.6
	surface density, m^2/cm^3	CMC	0.124±0.002	0.117±0.004	0.097±0.005**	0.111±0.004*
		CMC nuclei	0.008±0.001	0.009±0.002	0.0070±0.0005	0.0070±0.0004
		capillaries	0.040±0.006	0.034±0.004	0.030±0.002	0.034±0.005
23-month-old rats	volume density, mm^3/cm^3	CMC	808.8±10.9	765.5±50.1	803.6±28.4	811.5±28.2
		CMC nuclei	8.0±2.3	6.2±1.3	7.7±2.3	9.8±2.0
		capillaries	70.1±11.9	72.7±7.7	67.1±7.3	63.4±1.2
		endothelial cells	6.1±0.5	9.5±1.1*	5.3±1.3	5.3±0.3
		connective tissue cells	5.9±0.9	2.0±0.4*	6.1±1.8	8.4±3.4
		fibers and basic substance of the connective tissue	109.1±16.7	150.3±45.8	118.0±21.9	111.5±33.1
	surface density, m^2/cm^3	CMC	0.111±0.001	0.106±0.006	0.100±0.003**	0.082±0.014
		CMC nuclei	0.005±0.001	0.0040±0.0005	0.0040±0.0008	0.0050±0.0008
		capillaries	0.031±0.001	0.038±0.002*	0.038±0.003*	0.031±0.004

TABLE 3. Secondary Stereological Parameters of Myocardial Tissue in Wistar Rats after Injection of Doxorubicin in a Single Dose of 5 mg/kg (*M*±*m*)

Parameter			Control	Time after doxorubicin injection, days		
				5	14	21
1-month-old rats	surface/volume ratio, m^2/cm^3	CMC	0.155±0.004	0.147±0.011	0.119±0.006*	0.141±0.003
		CMC nuclei	0.709±0.072	0.638±0.061	0.568±0.052	0.654±0.043
		capillaries	0.574±0.004	0.544±0.016	0.595±0.029	0.576±0.044
		capillaries/CMC	0.050±0.008	0.042±0.006	0.037±0.002	0.043±0.007
	volume ratio (number)	nucleus/cytoplasm	0.016±0.004	0.019±0.006	0.0160±0.0006	0.014±0.001
		capillaries/CMC	0.088±0.012	0.078±0.012	0.062±0.003	0.074±0.008
		stroma/parenchyma	0.253±0.022	0.262±0.050	0.232±0.026	0.271±0.030
23-month-old rats	surface/volume ratio, m^2/cm^3	CMC	0.137±0.004	0.138±0.001	0.125±0.002*	0.100±0.014*
		CMC nuclei	0.651±0.053	0.714±0.092	0.575±0.062	0.533±0.033
		capillaries	0.460±0.062	0.535±0.035	0.561±0.016	0.487±0.072
		capillaries/CMC	0.038±0.002	0.051±0.005	0.047±0.006	0.038±0.006
	volume ratio (number)	nucleus/cytoplasm	0.010±0.003	0.008±0.002	0.010±0.004	0.012±0.002
		capillaries/CMC	0.086±0.015	0.097±0.014	0.084±0.013	0.078±0.001
		stroma/parenchyma	0.237±0.017	0.318±0.091	0.247±0.046	0.234±0.043

Structural reorganization of the myocardium in old rats was manifested in an increase in the surface density of capillaries on days 5 and 14 (by 23%). These changes contribute to an increase in the surface/volume ratio of capillaries (by 16 and 22%, respectively). We also revealed an increase in the surface/volume ratio of capillaries-to-CMC (by 34 and 24%). The volume and surface density of endothelial cells increased significantly by the 5th day (by 56 and 67%, respectively), but decreased on days 14 and 21 (by 13-17%). By contrast, the volume and surface density of connective tissue cells decreased by the 5th day (by 66 and 50%, respectively), but increased on days 14 and 21 (by 42-50%). The stroma/parenchyma volume ratio was elevated only on day 5 (by 34%). It was associated with an increase in the volume density of fibers and basic substance of the connective tissue.

Comparative study of structural reorganization of the myocardium in rats with anthracycline-induced regenerative and plastic insufficiency showed that cardiac remodeling during various periods of ontogeny is mediated by different cellular mechanisms. Differences were found in proliferative activity of CMC and ability of these cells for the hypertrophic growth. High proliferative activity of CMC in the heart of young animals contributes to the maintenance of cell population. Young animals are characterized by the accelerated physiological growth of CMC. This process is followed by the hypertrophic growth, which determines an increase in the weight of the heart (by 43%). The development of regenerative and plastic insufficiency of the heart in old animals is accompanied by a significant decrease in the total number of CMC (by 20% during various periods of the study). The degree of CMC hypertrophy is highest in old animals. The reduction of cell number is accompanied by a decrease in the weight of the heart (by 25%). It should be emphasized that dilation remodeling of the heart is observed in animals of various age groups and may be considered as a terminal state.

Single exposure to cardiotoxic factors during early ontogeny is followed by a strong activation of the regenerative and compensatory and adaptive reactions in CMC. These changes determine a rapid exhaustion of the regenerative potential in CMC and contribute to preterm "age-related" remodeling of the myocardium (aging).

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