Proliferative Activity of Cadriomyocytes in Chronic Hypercholesterolemia

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We studied proliferative activity of cardiomyocytes (using proliferation marker Ki-67) and compared it with their total number in the heart under conditions of experimental chronic hypercholesterolemia and its combination with hypothyroidism. It was found that Ki-67-positive cells are primarily located in the subepicardial layer near the heart base in both intact and experimental animals. Replicative cardiomyocyte pool in intact rats constituted 1.67±0.33‰ of the total cardiomyocyte population; after 68-day atherogenic diet with exogenous cholesterol alone, the replicative cardiomyocyte pool decreased by 16% (to 1.40±0.24‰). Treatment with mercazolil against the background of exogenous cholesterol increased this parameter by 40% (to 2.33±0.88‰). Changes in replicative activity of cardiomyocytes correlated with their total number in the heart and organ weight. We conclude that replicative cardiomyocyte pool primarily includes non-terminally differentiated cardiomyocytes (small mononuclear cardiomyocytes) and their proliferation maintains the total number of cardiomyocytes in the heart under conditions of cytopathic influences and provides the basis for physiological and reparative regeneration of the myocardium.

Key Words: *hypercholesterolemia; hypothyroidism; proliferative activity of cardiomyocytes; immunocytochemistry*

Proliferative activity of cardiomyocytes (CMC), *i.e.* cell regeneration, under various physiological and pathological conditions is the basis of physiological and reparative regeneration of the heart providing its structural integrity and functional activity [3,13,19]. During recent decades, the possibility of CMC proliferation after myocardial damage of different genesis was proven in clinical and experimental studies due to introduction of new technologies of visualization of molecular processes related to cell replicative activity into morphological studies. These studies provided the basis for the development of technologies of cell therapy aimed at restoration of lost myocardial weight and prevention of terminal stage of cardiac insufficiency.

Methods of visualization of proliferative (replicative) activity of CMC, apart from detection of mitosis phases, include immunocytochemical assay based on the use of monoclonal or polyclonal antibodies to nuclear proteins expressed during DNA replication (Ki-67, PCNA) or to halogenated thymidine analogs (5-bromo-2'-deoxyuridine and 5-iodo-2'-deoxyuridine). Proliferative activity of CMC was evaluated by their total number in the heart in the postnatal ontogeny and during the experiment [1]. Comparison of proliferative activity evaluated by histochemical methods and quantitative estimates of CMC population (alkaline and enzymatic dissociation of the heart tissue) reflects the contribution of proliferative reactions of CMC into heart regeneration and the ratio of cell death and proliferation.

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Hypercholesterolemia (HCH) is considered as a pathogenic factor inducing atherogenic vascular remodeling (formation of atherosclerotic plaques) and dysfunction of endothelial cells and vascular smooth muscle cells. Damage to the vascular intima caused by endogenous and exogenous factors is accompanied by accumulation and modification of apolipoprotein B on the surface of the vascular wall, activation and proliferation of endothelial cells, migration and activation of inflammatory cells, in particular, macrophages, and proliferation of smooth muscle cells [18]. These molecular and cellular modifications lead to the formation of atherosclerotic plaques; progressive growth of these plaques in coronary arteries can eventuate in partial or complete vascular occlusion and thromboembolism.

Proliferative and functional activity of arterial endotheliocytes and smooth muscle cells in HCH is affected by some mitogenic factors synthesized by inflammatory cells, in particular, macrophages, migrating into the inflammatory focus [20]. Accumulation of fatty acids in the myocardium is also associated with some metabolic, structural, and electromechanical changes in CMC and development of so-called lipotoxic cardiomyopathy [16]. Accumulation of triglycerides in CMC due to disturbances in their oxidation can induce cell death and lead to the development of heart failure. The intensity of regenerative responses of CMC, e.g. their proliferation, under these conditions considerably influences the development of the pathological process and its outcome.

The objective of the present study was to evaluate the replicative pool (proliferative activity) of CMC under experimental hypercholesterolemia induced by atherogenic diet and hypothyroid status.

MATERIALS AND METHODS

HCH was modeled in male Wistar rats weighing 390-560 g; the animals were divided into two experimental groups. Group 1 animals (n=6) received atherogenic diet: standard laboratory chaw supplemented with cholesterol (25 mg/100 g body weight; Panreac Quimica SA); group 2 rats received atherogenic diet in combination with antithyroid preparation mercazolil (25 mg/100 g, Akrikhin). The animals were fed according to the following scheme: days of atherogenic diet were alternated with days of starvation; water was given every day *ad libitum*. The control group (n=6) was maintained under standard conditions and received standard food every day. The experiments were performed with strict adherence to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes.

The animals were sacrificed on day 68 of the experiment. Myocardial specimens were fixed in 10% neutral formalin and embedded in paraffin. Proliferative activity of CMC was evaluated immunohistochemically using proliferation marker Ki-67; an indirect two-step assay was used (primary antibodies and visualization system from purchased from Spring). Primary antibodies were diluted 1:100, DAB was used as the chromogen. The specimens were examined under a Leica DM 4000B microscope and photographed using a Leica DFC 320 camera; the images were processed using Leica QWin software. Index of Ki-67-labeled CMC was evaluated by counting 1000 cells; CMC diameter was evaluated using Leica QWin software (at least 200 cells were analyzed).

Quantitative evaluation of the total population of CMC in the heart was performed after alkaline dissociation of the fixed tissues [1]. The data were processed statistically using Statistica 6.0 software; significance of differences in case of normal distribution was evaluated by Student's t test.

RESULTS

The used models of HCH induced changes in the blood lipid spectrum and these changes were more pronounced in animals receiving atherogenic diet in combination with mercazolil [4]. Total plasma cholesterol content increased in animals of groups 1 and 2 by 9 and 38%, respectively (p<0.05, Fig. 1, *a*), LDL by 10 and 47% (p<0.05), and HDL by 9 and 32% (p<0.05).

Chronic HCH, especially in combination with hypothyroidism induced a spectrum of structural and functional changes in CMC (lytic changes of myofibrillar bundles of different severity, lipid infiltration, partial necroses, *etc.*) In both experimental groups, the major changes in the myocardium were lytic changes in CMC of different severity (with signs of perinuclear devastation) and reactive changes in the stroma and vessels. In some CMC, vacuole-like formations were seen (honeycomb sarcoplasm). In both experimental groups, considerable polymorphism of CMC nuclei was observed, CMC contractures were less incident. Many CMC contained small lipid inclusions (scattered or arranged in chains). Alimentary HCH combined with hypothyroidism was associated with more pronounced lytic and necrobiotic changes in CMC. In both experimental groups, macrophages containing lipid inclusions were found in the myocardium; they can be considered as structural and functional analogs of foam cells in atherosclerotic plaques – myocardial (interstitial) foam cells.

Considerable decrease in the heart weight (by 25%, p<0.01) detected on day 68 in group 1 rats (Fig. 1, *b*) was determined by a decrease in the total number of CMC (by 21%, Fig. 1, *c*), because the mean CMC diameter remained practically unchanged (15.97±0.23

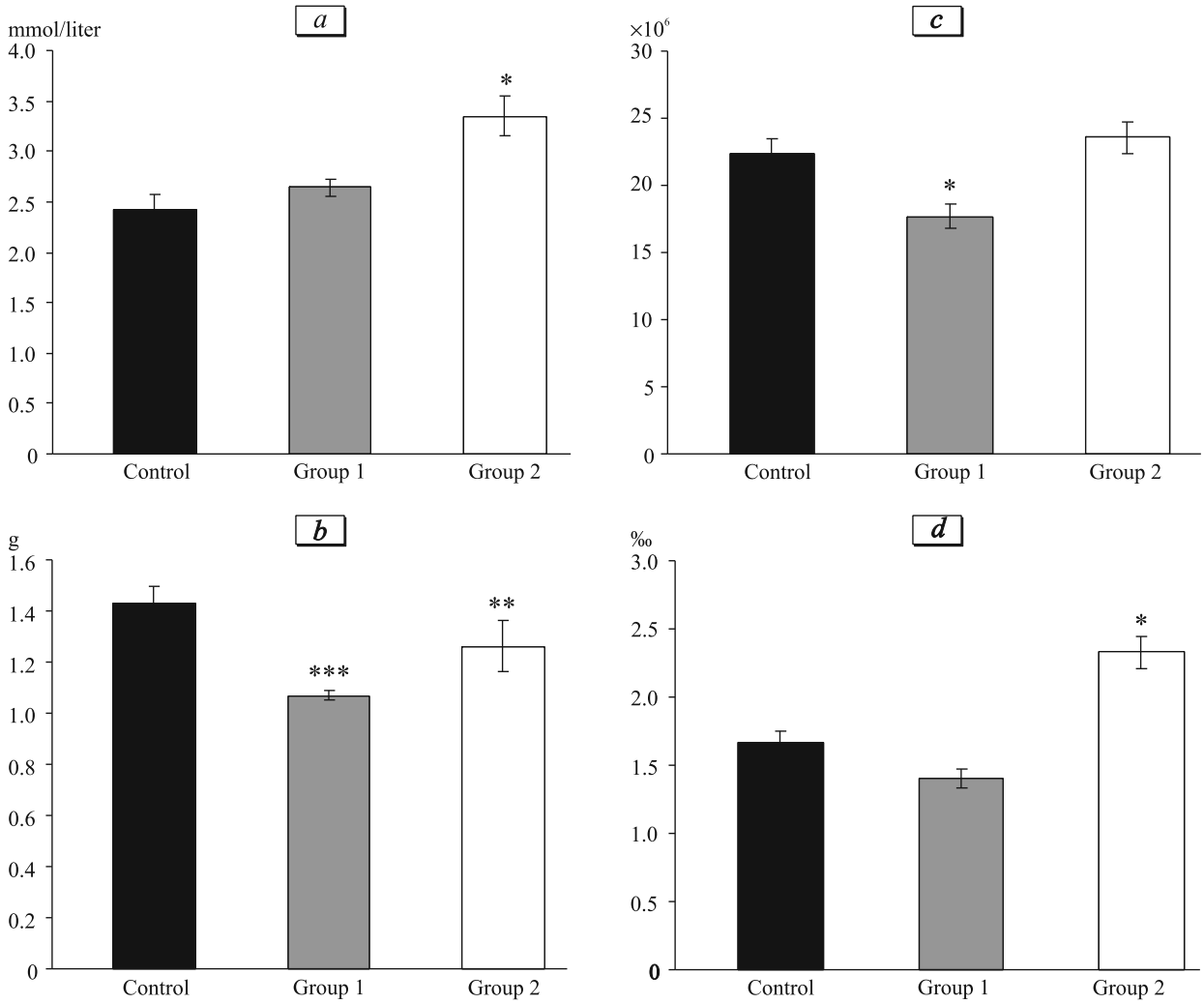

Fig. 1. Blood cholesterol content, heart weight, quantitative changes in CMC population and their replicative activity during HCH modeling. *a*) total cholesterol, *b*) heart weight, *c*) total number of CMC, *d*) percentage of Ki-67-positive CMC. *p<0.05, **p<0.01, ***p<0.001 in comparison with the control.

vs. 16.02±0.51 μ in the control). In group 2 rats, the heart weight was reduced insignificantly (by 12%), which was explained by the absence of pronounced changes in CMC diameter (15.38±0.41 μ) and their total number (Fig. 1, *c*).

Immunocytochemical analysis showed that Ki-67-positive CMC in the myocardium of control rats were primarily located near the heart base and in the subepicardial layer. Proliferative activity of CMC was recorded rarely in the medium layer of the myocardium and was absent in the subendocardial layer. In the myocardium of controls, Ki-67-positive CMC constituted 1.67±0.33‰ (Fig. 1, *d*). CMC expressing Ki-67 were presented by the following two phenotypes: small CMC (diameter 7-9 μ, length 36-42 μ), mononuclear and sometimes, binuclear (late-telophase) cells and CMC of regular size (diameter 14-15 μ, length 70-80 μ), mononuclear and rare binuclear cells.

In the myocardium of group 1 rats, Ki-67-positive CMC were also primarily located near the heart base in the subepicardial and medium layers. These were primarily small mononuclear CMC (Fig. 2, *a, b*) or rarely binuclear cells (Fig. 2, *c*) with two Ki-67-positive nuclei. Eating of exogenous cholesterol alone (group 1) led to a decrease in the percentage of Ki-67-positive CMC by 16% (to 1.40±0.24‰, Fig. 1, *d*). This reduction correlated with a decrease in the total number of CMC in the heart. In group 2 rats, the content of Ki-67-positive CMC increased by 40% (to 2.33±0.88‰, Fig. 1, *d*). This contributed to the maintenance of the total number of CMC in the heart.

Evaluation of replicative activity of CMC based on immunocytochemical detection of Ki-67-positive CMC yielded different results depending of animal species, age, sex, and the presence of acute and chronic pathological processes affecting the functional ac-

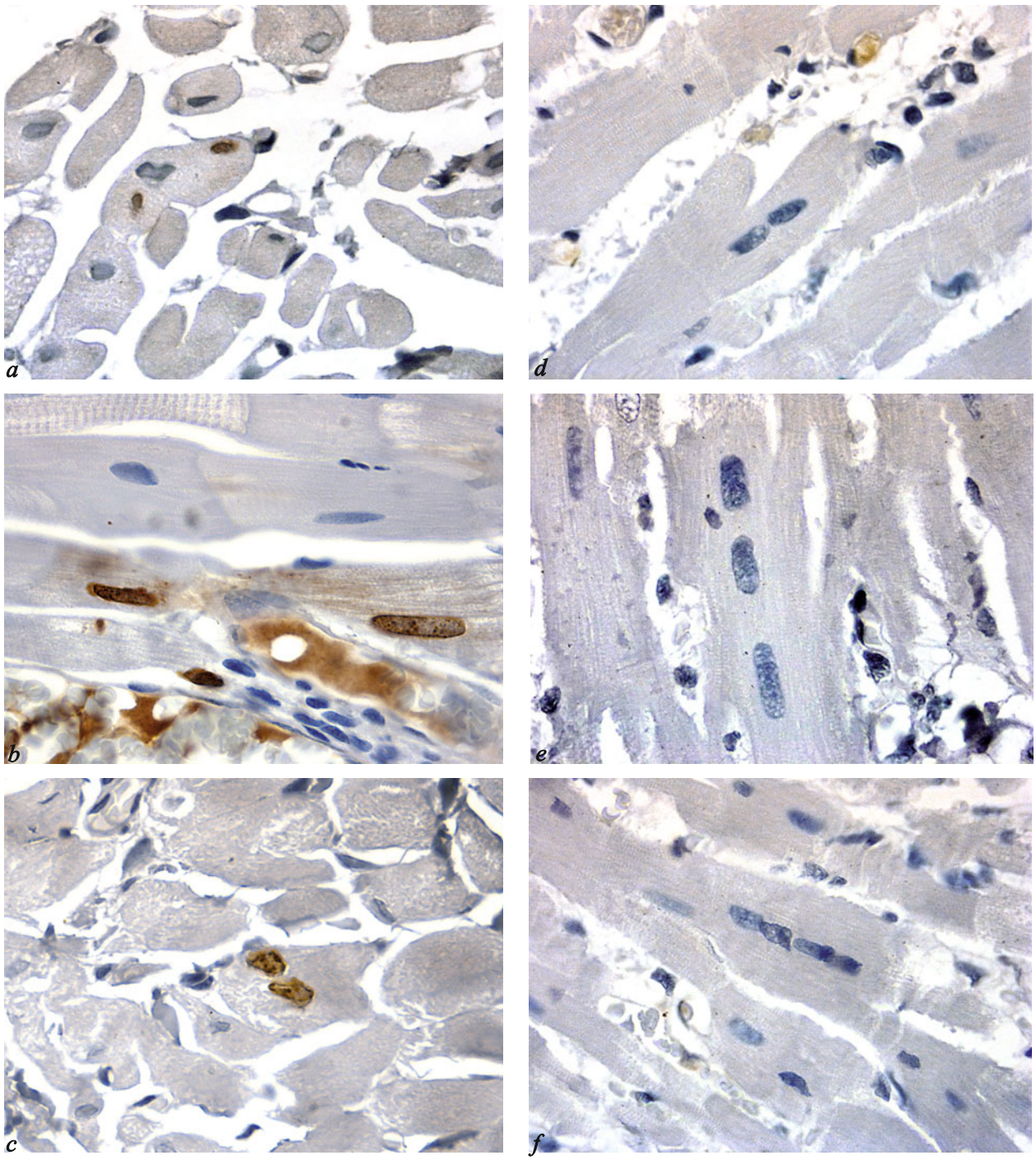

Fig. 2. Expression of Ki-67 in rat CMC under conditions of experimental chronic HCH. Immunocytochemical staining, ×1000. *a*) Ki-67 in nuclei of subepicardial CMC, *b*) Ki-67-positive mononuclear CMC around a microvessel, *c*) binuclear CMC with KI-67-positive nuclei, *d*) small binuclear CMC; *e*) small trinuclear CMC, *f*) small tetranuclear CMC.

tivity of the myocardium. For instance, the number of Ki-67-positive CMC in the myocardium of 3-month-old female rats is 0.0057% and increases to 0.0179% (by 216%) under the effect of growth hormone [5], which is significantly lower than in males. Sex-related differences in replicative activity of CMC determine differences in the compensatory and adaptive remodeling of the heart in females and males (predominance of CMC hypertrophy and hyperplasia, respectively) in response to adverse exposure [7]. Evaluation of replicative activity of CMC in the myocardium with bromodeoxyuridine provided similar results: 0.1-0.3% of the total number of CMC [7]. In the hypertrophic mouse myocardium, labeling with thymidine demon-

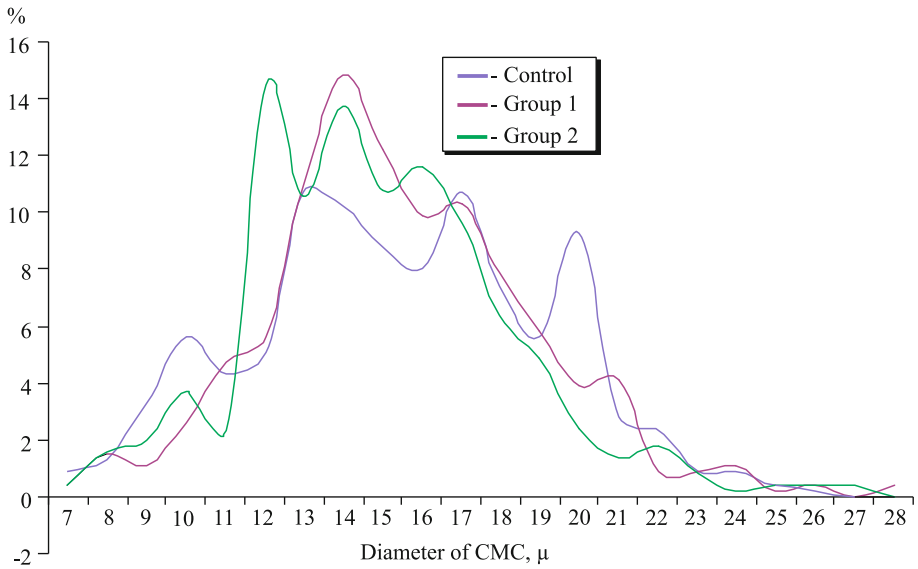

Fig. 3. Uniform distribution of CMC under conditions of experimental HCH.

strated lower proliferative activity of CMC (0.0014% of the total number of CMC) [17].

In donor heart without pathological changes (unsuitable for transplantation for some reasons), the number of Ki-67-positive cells was 3.92±0.99%; in hypertension and cardiomyopathy of different types (including alcoholic cardiomyopathy), expression of Ki-67 increased by 2.5-4.0 times [11]. In endocardium biopsy specimens obtained from heart transplantation patients with acute rejection episodes, the number of Ki-67-positive CMC was 7.6±2.2% (vs. 0.8±0.2% in myocardium without pathological processes) [14]. These findings suggest that pathogenic and stress factors producing a damaging effect on the myocardium largely stimulate CMC proliferation.

Detection of Ki-67-positive CMC in the myocardium of intact animals and during HCH modeling and comparison of these values with the data on the total number of CMC in the heart suggest that the replicative pool consists of preexisting, so-called non-terminally differentiated (immature) CMC (small CMC). Small mononuclear CMC more often than large binuclear CMC incorporate bromodeoxyuridine (3.1% *vs.* 0.8%), do not express aging protein $p16^{INK4a}$, and are characterized by higher telomerase activity and T-type calcium current typical of fetal neonatal CMC [6].

These findings agree with the data of other researchers who demonstrated that physiological and reparative regeneration of the myocardium during aging, in the postinfarction period, and under the effect of cytostatic (paclitaxel) occurs through proliferation of preexisting CMC, rather than progenitor cells migrating to the myocardium [12,15]. This conclusion was confirmed by the localization of Ki-67-positive CMC at the ends muscle fibers with the formation of growth points and in the middle of muscle fibers, which provided intermediate growth. At the same time, replicative activity of CMC and functional activity of inflammatory cells in the damage foci (especially, in the peri-infarction zone) can intensify migration of progenitor cells to these zones and stimulate CMC proliferation and regeneration of the myocardium [12].

Immunocytochemical detection of Ki-67 predominantly in the nuclei of small mononuclear CMC allows considering these cells as the main population providing CMC proliferation and appearance of bi- and multi-nuclear cells (Fig. 2, *d-f*). Analysis of CMC diameter variability revealed polymodality of their size distribution in the control and in both experimental groups (Fig. 3), which attests to the presence of several (~3) cell subsets in CMC population that differ by their morphofunctional characteristics. Under conditions of HCH, the number of small CMC increased and the peaks of the size distribution curve were shifted to the left.

The populations of small mono- and binuclear CMC are not static and can change under the influence of various factors. We have previously demonstrated that cytotoxic influences (cyclophosphamide and doxorubicin treatment) change the percentage of mono- and binuclear CMC and the number of small CMC in the myocardium [1,2]. The percent of mononuclear cells on days 3 and 14 after doxorubicin administration decreased by 20 and 34%, respectively, and after cyclophosphamide administration by 31 and 13%, respectively. In contrast, the number of small CMC (both mono- and binuclear cells) increased on day 14 after administration of cytotoxic drugs (by 2 times after administration of cyclophosphamide and by 36% after administration of doxorubicin). Simultane-

ous administration of chemical compounds correcting the cardiotoxic effects of cytostatics (particularly betulonic acid and its derivatives) led to an increase in the proportion of mononuclear CMC, which was accompanied by an increase in total number of CMC in the heart [1].

The concept of small mononuclear CMC as the main cell population forming the replicative pool raises the question about prevention of possible artifacts in their identification. Since the length of CMC depends on contraction/relaxation of its myofibrils, CMC with contracture injuries should not be erroneously ascribed to this pool. Detailed analysis of tinctorial and structural features (among other methods, by polarization microscopy) of small CMC allows their classification as immature cells (high acidophilia, reduced number of sarcomeres, increased time f contraction and of Ca^{2+} redistribution), the number of which varies during ontogeny and under the action of cytopathic agents [2,6]. Increased percentage of mononuclear shortened (with reduced number of sarcomeres) CMC (from 11.6 to 18%) after modeling of chronic volume load has been demonstrated by other researchers [7].

Thus, modeling of chronic HCH using different atherogenic diets modified replicative activity of CMC in different ways. Administration of exogenous cholesterol for 68 days led to a decrease in proliferative activity of CMC followed by reduction of the total number of CMC in the heart and more pronounced decrease in heart weight. Combined administration of exogenous cholesterol and mercazolil for 68 days led to an increase in proliferative activity of CMC, due to which their total number remained unchanged and the loss of heart weight was less pronounced. The replicative pool includes preexisting non-terminally differentiated CMC (small mononuclear CMC); proliferation of these cells maintains the total number of CMC in the heart.

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