# Pharmacological Regulation of Functional Activity of Stem Cells in Restoration of the Myocardium during the Postinfarction Period

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The effect of granulocytic CSF on myocardial tissue recovery after acute myocardial infarction was studied on rats. A course of granulocytic CSF after ligation of the left coronary artery normalized ECG parameters and morphological picture of the myocardium 1 month after treatment. It was shown on mouse model of myocardial infarction that this process was associated with more intensive mobilization and homing of mesenchymal stem cells in the heart.

**Key Words:** *myocardial infarction; regeneration; stem cells*

Coronary heart disease (CHD) is the most incident cardiovascular disease in adults. CHD and its acute form (myocardial infarction) often lead to cardiosclerosis and heart failure (HF) [1]. Modern methods for prevention and treatment of HF, making use of a wide armory of drugs and nonmedicamentous means, remain little effective, because they cannot arrest progressive development of HF and do not reduce its incidence [11]. This is due to the fact that none of the methods used in practical cardiology eliminates the main cause of HF development: replacement of myocardial tissue with noncontracting connective tissue [7].

Recently cell therapy of HF received great attention as a method aimed at restoration of functional activity of the myocardium by means of stem cell transplantation [9,14,15]. On the other hand, new methods for mobilization of the recipient own bone marrow mesenchymal stem cells (MSC) by injection of specific mobilization factors, *e. g.* granulocytic CSF (G-CSF) and stem cell factor, are intensively developed now. Positive results of preventive and therapeutic injections of these factors to animals with experimental myocardial infarction were reported [2,12,13].

We studied the effect of G-CSF on myocardial recovery during the postinfarction period after administration of G-CSF preparation to rats with experimental acute myocardial infarction.

## MATERIALS AND METHODS

Experiments were carried out on 36 male Wistar rats (250-300 g) and 52 CBA/CaLac mice (18-20 g). Certified conventional 1st category animals were obtained from Breeding Center of Institute of Pharmacology. Experimental myocardial infarction was modeled in rats by thoracotomy followed by ligation of the left coronary artery at the level of the first quarter of distance from the pulmonary cone to the heart apex under ether narcosis with visual and ECG control. After surgery the rats with ECG signs of ischemic heart injury were divided into 2 equal groups. Experimental animals were injected with G-CSF (preparation was developed by Vektor

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Company in collaboration with Institute of Pharmacology [15]) in a dose of 100 mg/kg subcutaneously in 0.5 ml saline once daily for 5 days. The first injection was made 3 h after surgery. Controls received normal saline (0.5 ml) according to the same protocol.

In mice myocardial infarction was induced by intravenous injection of epinephrine (0.25 mg/kg) 24 h before the first injection of G-CSF using a previously described method [5] in our modification. CSF was injected in the same dose according to the same protocol in 0.2 ml saline. Experimental material was collected from intact and experimental animals on days 3, 7, 10, and 14.

ECG was recorded in all rats before surgery and 3 h and 30 days after coronary artery ligation. On day 30 of the experiment the animals of both groups were sacrificed by ether overdose, the chest was opened, and the heart was removed. For morphological examination the myocardium (without aorta, the right ventricle, ventricular septum) was placed into 10% formalin. After routine dehydration the left ventricle was embedded in paraffin. Sections (5 μ) of the entire left ventricle (from the base to the apex) were made with 300-μ steps. The preparations were stained with picrofuchsin (for detecting connective tissue) and photographed (the entire area) using a microvideocam. The ratio of structural elements of the myocardium was evaluated using computer software for graphical data processing.

The content of MSC in the bone marrow and peripheral blood was measured by the method of limiting dilutions [10]. The bone marrow was washed from the femoral bone with 0.5 ml preparative medium (95% RPMI-1640, 5% FCS), suspended, and total count of myelokaryocytes was determined by evaluating their viability. The peripheral blood was collected under sterile conditions from cervical vessels after partial decapitation under ether narcosis [3]. The blood was collected into centrifuge tubes with a medium containing 100 U/ml heparin (Biochemie) and mononuclear fraction was isolated by density gradient centrifugation (Histopaque-1077, Sigma).

Cell material was placed into culture medium containing heparin (Biochemie) and basic fibroblast growth factor (Sigma); 8 variants of cell concentrations were prepared: from the maximum (750,000 cell/ml) to the minimum (2500 cell/ml). All samples were then placed into plastic 96-well plates (Costar) coated with 1% gelatin and incubated for 6 weeks in a $CO_2$ incubator (Jouan) at 37°C, 5% $CO_2$, and 100% humidity; the medium was changed twice a week. After incubation the number fibroblast-like cells per well was determined. If fibroblast content in the well was equal or surpassed 10, the well was evaluated as "positive", if it was below 10 — as "negative". Rows reflecting the presence or absence of parental cells in different dilutions of the initial cell material were thus obtained. The incidence of MSC was evaluated using generalized linear model for Poisson distribution. Correspondence of the limiting dilutions data to the Poisson unidimensional model was evaluated by linear log-log regression [10].

The content of clonogenic cells in the myocardium was evaluated by cell cloning in liquid culture medium (in our modification). The heart was washed twice from erythrocytes in normal saline, the myocardium was fragmented and put for 10 min into EDTA solution containing 1.25 mg/ml trypsin at ambient temperature. The material was then dissociated to a cell suspension, first using a syringe with a 2-mm needle (5 min) and then with a homogenizer. The cell suspension was filtered through a capron mesh in order to remove large aggregations, washed twice by centrifugation at 1500 rpm for 5-10 min, and the total count of nucleated cells was evaluated by determining their viability (trypan blue staining). The cells were put into culture medium supplemented with heparin (Biochemie), insulin (Novo Nordisk), stem cell factor (Sigma), IL-6 (Sigma), basic fibroblast growth factor (Sigma), and endothelial growth factor-β. The concentration of cell elements was brought to 200,000 per 1 ml, after which the resultant suspension was transferred into 24-well plates (0.5 ml per well). The culture was incubated for 7 days at 37°C, 5% $CO_2$, and 100% humidity. After incubation colonies containing myocyte-like cells were counted under an invert microscope. Aggregates containing more than 30 cells were considered as colonies.

The results were processed by methods of variation statistics using Statistica 6.0 software.

## RESULTS

The mortality of rats during the early periods after coronary artery occlusion (on day 2) was 34%. Later all animals treated with G-CSF survived; 6% control animals died, presumably because of progressive HF.

The initial ECG values were virtually the same in experimental and control groups and within the normal range of values for these animals [4]. Pronounced ECG changes were observed 3 h after ligation of the coronary artery (Fig. 1). The amplitude of wave *T* sharply increased, indicating the development of significant ischemic disorders in the myocardium. In parallel, the amplitude of *R*

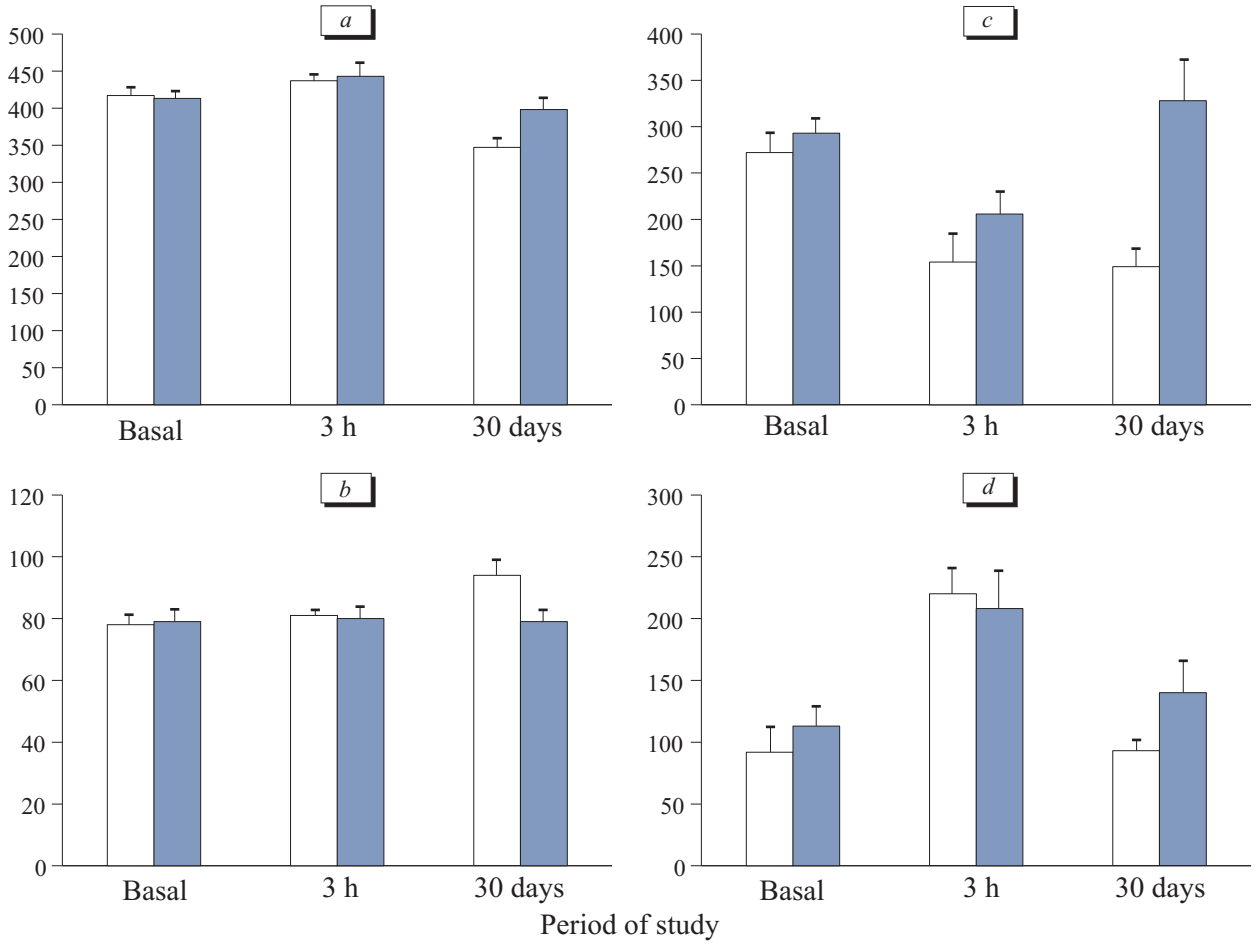


**Fig. 1.** Electrocardiogram values in rats with experimental myocardial infarction receiving no treatment (light bars) and treated by G-CSF (dark bars). *a*) heart rate, $min^{-1}$; *b*) *QT*, msec; *c*) *R*, μV; *d*) *T*, μV.

wave decreased, and a pathological *Q* wave emerged in 42% animals. The *QRS* complex was absent in many cases and a *QT* complex formed. These changes in the *QRS* complex reflected the formation of a vast necrotic zone in the myocardium. These shifts in ECG parameters indicate the development of acute stage of myocardial infarction [6].

The *T* wave returned virtually to normal in rats of both groups 30 days after ligation of the coronary artery, this indicating resolution of the ischemic zone. In control rats *R* wave amplitude remained low (140±26 μV). Heart rate also decreased significantly (347±13 $min^{-1}$) and ventricular "electric systole" was delayed (lengthening of *QT* interval). A pathological *Q* wave was detected in one animal. These ECG changes indicate postinfarction cardiosclerosis (cicatricial stage of myocardial infarction) in control rats.

The amplitude of *R* wave in animals treated with G-CSF reached 328±44 μV by day 30 after coronary artery ligation, which was significantly higher than in the control group. Heart rate was also 13% higher, and statistically significant shortening of the *QT* interval was observed. Hence, ECG improved in this group in comparison with the control; moreover, there were no appreciable differences from ECG values before myocardial infarction modeling (Fig. 1).

Hence, the course of G-CSF normalized ECG parameters in rats with ligated coronary artery by day 30 of the experiment.

Histological study showed the formation of a connective tissue cicatrix at the site of necrotic zone 30 days after infarction modeling in experimental animals of both groups. In control group virtually the entire heart wall in the cicatrix zone was presented by the connective tissue. The percentage of connective tissue elements in the total myocardial area on the preparations was 4.43±0.93%. In animals receiving G-CSF postinfarction sclerosis was less pronounced the cicatricial zone contained collagen fibers and cardiomyocytes, occupying only 0.39±0.15% of total myocardial area.

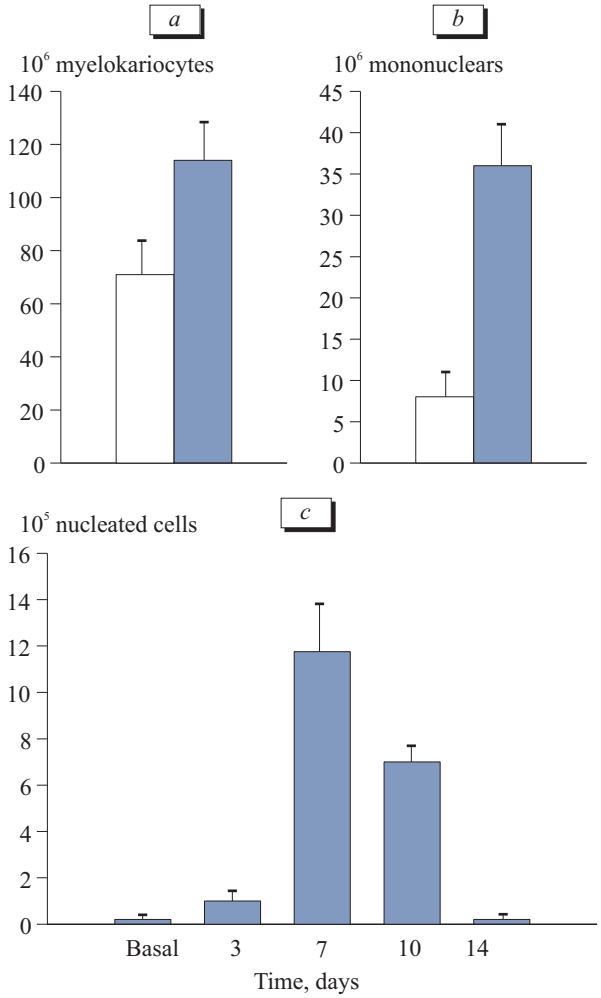


**Fig. 2.** Content of mesenchymal stem cells in the bone marrow (*a*), peripheral blood (*b*) on day 3 of experiment and of clonogenic cells in heart tissue (*c*) over the course of experiment in mice with experimental myocardial infarction without treatment (light bars) and treated by G-CSF (dark bars).

The content of MSC in the peripheral blood increased significantly on day 3 after injection of G-CSF (Fig. 2), indicating their mobilization from the depot. Parallel increase in the bone marrow count of MSC suggests that stem cells are released into the blood not only from the bone marrow, but also from other sources. It seems that the cytokine activated the parental cells in the bone marrow.

The content of clonogenic cells in the myocardial tissue increased significantly on days 7 and 10 of the experiment (Fig. 2) and returned to the basal level on day 14 after the start of G-CSF treatment. Microscopic examination showed cell groups of 30 and more cells with the morphology of fibroblasts, among which few myocyte-like elements were seen (Fig. 3).

These data confirm the hypothesis on accumulation of clonogenic cells at the site of injury and

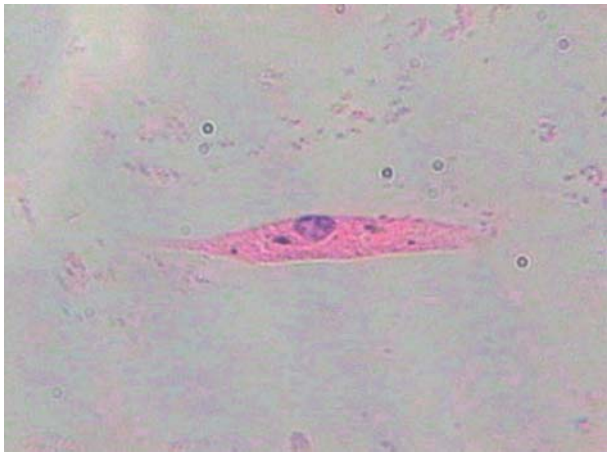

**Fig. 3.** Myocyte-like cells in culture of cardiac clonogenic cells.

their further differentiation into functioning myocardial cells after mobilization of MSC under the effect of G-CSF. However, the appurtenance of these cells to this or that class of parental cells remains not quite clear: whether these cells are MSC migrating from the bone marrow and tissue depots, their committed descendants, or regional cardiomyocyte precursor cells.

Hence, the course of G-CSF mobilizing the bone marrow MSC [13] effectively stimulated regeneration processes in the myocardial tissue exposed to ischemic injury. After mobilization and homing the mesenchymal precursor cells are differentiated into working myocardial tissue, which prevents the development of connective tissue cicatrix and improves functional activity of the myocardium during the postinfarction period.

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