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The Antiapoptotic Effect of Granulocyte Colony-stimulating Factor Reduces Infarct Size and Prevents Heart Failure Development in Rats

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Key Words

G-CSF • Myocardial infarction • Apoptosis • Chamber dilation • Heart failure

Abstract

Background/Aim. Granulocyte colony-stimulating factor (G-CSF) reduces myocardial injury and improves cardiac function after myocardial infarction (MI). We investigated the early alterations provided by G-CSF and the chronic repercussions in infarcted rats. **Methods.** Male Wistar rats (200-250g) received vehicle (MI) or G-CSF (MI-GCSF) (50 µg/kg, sc) at 7, 3 and 1 days before MI surgery. Afterwards MI was produced and infarct size was measured 1 and 15 days after surgery. Expression of anti- and proapoptotic proteins was evaluated immediately before surgery. 24 hours after surgery, apoptotic nuclei were evaluated. Two weeks after MI, left ventricular (LV) function was evaluated, followed by in situ LV diastolic pressure-volume evaluation. **Results.** Infarct size was decreased by 1 day pretreatment before occlusion (36 ± 2.8 vs. $44 \pm 2.1\%$ in MI; $P < 0.05$) and remained reduced at 15 days after infarction (28 ± 2.2 vs. $36 \pm 1.4\%$ in MI; $P < 0.05$). G-CSF pretreatment increased Bcl-2 and Bcl-xL protein

expression, but did not alter Bax in LV. Apoptotic nuclei were reduced by treatment (Sham: 0.46 ± 0.42 , MI: 15.5 ± 2.43 , MI-GCSF: $5.34 \pm 3.34\%$; $P < 0.05$). Fifteen days after MI, cardiac function remained preserved in G-CSF pretreated rats. The LV dilation was reduced in MI-G-CSF group as compared to MI rats, being closely associated with infarct size. **Conclusion.** The early beneficial effects of G-CSF were essentials to preserve cardiac function at a chronic stage of myocardial infarction.

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Introduction

Experimental myocardial infarction (MI) has been widely used to study the development of heart failure. Post-infarction cardiac remodeling leads to structural and molecular changes throughout the myocardium, including both ischemic and non-ischemic areas. One of the key markers of cardiac failure after infarction is left ventricular (LV) enlargement, which is mainly determined by the infarct size but also by slippage of myocyte strands leading to wall thinning and chamber dilation [1, 2]. Apoptosis is

an important type of cell death, and it contributes to the increase in infarct size during the acute phase of MI [3]. These early changes lead to severe hemodynamic impairments such as increased LV filling pressure and pump failure [4], representing a major cause of long-term mortality in infarcted animals [5].

Granulocyte colony-stimulating factor (G-CSF) is a 20 kDa polypeptide with hematopoietic actions that is produced by several cell lineages. Recently, its ability to mobilize bone marrow-derived stem cells has led some authors to test the potential effect of this cytokine in heart failure therapy [6]. Orlic et al. [7] showed that pre-treatment with G-CSF and stem cell factor (SCF) in mice attenuated post-infarction cardiac remodeling and improved myocardial function and survival, possibly by cardiac regeneration. Others have also observed cardiac tissue regeneration after MI with G-CSF therapy [8, 9]. This effect, however, was not found in other studies. Deten et al. [10] showed that pre-treatment with G-CSF plus SCF did not improve cardiac function after infarction and did not observe any evidence of cardiac tissue regeneration.

Some studies have shown that G-CSF alters the course of LV remodeling. In a swine model of MI, G-CSF treatment reduced infarct size and improved cardiac function [11]. In addition, Sugano et al. [12] observed that the acceleration of the healing process in the infarct zone mediated by G-CSF treatment led to decreased ventricular expansion in infarcted rats. It was also demonstrated that long-term use of low-dose G-CSF improved cardiac function [13]. It has not yet been determined, however, if the effects of G-CSF in the acute phase of myocardial infarction are effective in altering post-infarction remodeling. Thus, our purpose in the present study was to test the effects of G-CSF in the acute infarct phase and its repercussions for the development of heart failure.

Materials and Methods

Animals and experimental groups

Male Wistar rats (200-250 g) were randomly selected to receive three doses of G-CSF (Biosintetica, 50 µg/kg, subcutaneous) or vehicle (saline) at 7, 3 and 1 days before surgical procedures. Pre-treated animals (control and G-CSF) were subjected to coronary artery ligation to produce MI twenty-four hours after the final G-CSF treatment. Sham surgeries (SO) were performed in a sample of animals to be used as non-infarcted controls. A subset of animals from each group was killed before any surgical manipulation to determine

the effects of G-CSF treatment in the apoptosis-related proteins in the heart. During the pre-treatment period and after surgery, all animals were maintained in a 12-hour light-dark cycle and had free access to water and rodent chow. The experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and approved by the institutional animal research committee.

White blood cell counting

Blood samples (20 µL) were collected by puncture of the safena vein before the first dose and immediately before the surgical procedure to produce MI or SO and were homogenized in a lysis solution containing 2% acetic acid (380 µL). White blood cells (WBC) were quantified in a Neubauer chamber.

Western blot

A sample of animals (six male pre-treated with G-CSF and six pre-treated with vehicle) was killed twenty-four hours after the last dose of G-CSF. The animals were killed by decapitation, and the heart was rapidly removed and rinsed in cold saline solution. The LV was separated and minced. A sample from the LV was weighed and homogenized in buffer (100 mM Tris (pH 7.4), 1 % SDS, 10 mM Na₃VO₄, 100 mM NaF, 100 mM Na₄P₂O₇, and 10 mM EDTA) and then centrifuged at 12000 g for 30 min at 4° C to remove the insoluble material. Protein concentration was determined by the Lowry method. Equal amounts of protein (75 µg) from each sample were treated with Laemmli buffer (BioRad Laboratories), resolved by 12% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Amersham Biosciences) by electroblotting. The membrane was blocked with Tris-buffered solution (10 mM Tris, 100 mM NaCl and 0.1% Tween 20) containing 5% of non-fat milk for 2 hours and then incubated overnight at 4° C with the appropriate primary antibody diluted in the block solution. The specific primary antibodies used were anti-Bcl-2 (dilution 1:750; Santa Cruz Biotechnology Inc., CA, USA), anti-Bax (dilution 1:750; Santa Cruz Biotechnology Inc., CA, USA), and anti-Bcl-xL (dilution 1:750; Santa Cruz Biotechnology Inc., CA, USA). The membranes were then incubated with the respective peroxidase-conjugated secondary antibody (Amersham Biosciences) for 1 hour. Visualization of specific protein bands was performed using the enhanced chemiluminescence reagent ECL Plus (Amersham Biosciences) with exposure to photographic film. Densitometry was performed using a computerized digital image system (Scion Image, National Institute of Health). Within the same membrane, α-tubulin protein expression was also determined (anti-α-tubulin, dilution 1:500; Zymed Laboratories, CA, USA), and its content was used as an internal control.

Surgical procedures and infarct size measurement

Myocardial infarctions were performed by an investigator blinded to the treatment groups, as previously described [14]. Briefly, under pentobarbital anesthesia (50 mg/kg, i.p.), a thoracotomy was performed at the level of the fourth intercostal space to expose the heart. The left coronary artery was permanently occluded with a 6.0 suture, and the heart was

immediately returned to the thorax. Sham-operated (SO) rats were subjected to all procedures except the coronary occlusion.

To determine the acute cardiac changes produced by G-CSF pre-treatment, a sample of animals was sacrificed twenty-four hours after surgery to evaluate infarct size. The left ventricle was incubated with 1% triphenyltetrazolium chloride (TTC) in a buffer solution at 37° C for 10 minutes. Under microscope visualization, the ischemic area was separated from non-ischemic tissue and measured by planimetry (ImageJ v. 1.43; National Institute of Health, USA). Another group of animals was studied two weeks after the surgery. The infarct scar was carefully separated from viable tissue under a microscope and weighed, and the areas occupied by the scar tissue and by the remaining LV muscle were measured by planimetry. Infarct size was reported as the percentage of the scar area within the total LV area [14].

TUNEL assay

Twenty-four hours after SO or coronary ligation surgery, a random sample (5 animals per group) was used for the evaluation of apoptosis in the left ventricular myocardium. The hearts were excised, perfused with formalin solution and embedded in paraffin. After embedding, 4 µm sections were cut.

A terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay was used to identify double-stranded DNA fragmentation. Each section was deparaffinized and rehydrated with graded xylenes and ethanols. Proteinase K was applied for 30 min to induce proteolysis. The endogenous peroxidase was inhibited with 3% hydrogen peroxide for 10 min. Detection of apoptosis was performed using a commercial kit (Roche Diagnostics, Indianapolis, IN, USA). The TdT reaction was carried out for 1 hour at 37° C, and then 3,3'-diaminobenzidine tetrahydrochloride chromogen was applied. Harris hematoxylin was used as a counterstain. Images were acquired using an optical microscope (DM2500, Leica, Wetzlar, Switzerland). TUNEL-positive cells were determined in the ischemic area by randomly counting 5 fields per section, and the data were expressed as a percentage of apoptotic nuclei to total nuclei.

LV function measurement and pressure-volume evaluation

Two weeks after the surgery, the animals were anesthetized with ketamine (Agener União, Brazil) and xylazine (Bayer, Brazil) (70/10 mg/kg, i.p.) to evaluate LV function. The right common carotid artery was separated from the surrounding connective tissue and catheterized. A heparinized polyethylene catheter (P50) connected to a pressure transducer (TRI 21, Letica Scientific Instruments, Spain) and a digital system (Powerlab/4SP ML750, ADInstrument, Australia) was then inserted into the LV and the readings was saved for later analysis. Heart rate (HR), left ventricular systolic (LVSP), end-diastolic pressure (LVEDP), and the maximum rate of pressure rise (+dP/dt) and fall (-dP/dt) were assessed.

After hemodynamic measurements, the heart was arrested with 3 M KCl, and a double-lumen catheter (P50 inserted into P200) was inserted into the LV through the aorta to determine

the in situ LV diastolic pressure-volume relationship to measure the dilation of the chamber [15]. The atrio-ventricular groove was occluded, and a small incision was made in the right ventricular free wall to avoid any compressor effect. Then, 0.9% NaCl solution was infused with an infusion pump (BI 200, Insight Equipamentos, Brazil) at 0.68 mL/min into P200 catheter, and intraventricular pressure was continuously monitored with the P50 catheter. Fluid was infused until the pressure reached 30 mmHg. Three curves were recorded for each heart for approximately ten minutes. LV dilation was obtained through the slope of pressure-volume curve [15].

Statistical analysis

All data are presented as the mean ± standard error of mean (SEM), and significance was set at $P < 0.05$. Comparisons between two means were made using the Student's *t*-test, and more than two groups were compared using one-way analysis of variance (ANOVA), followed by a Bonferroni correction. When data did not follow a Gaussian distribution, a Kruskal-Wallis test was used. A Pearson correlation was performed to test the association between dilation index and the infarct size. Linear regression analysis was used to obtain the linear slope and to calculate the left ventricular dilation from the pressure-volume curve.

Results

Mobilization of WBC

All groups showed similar white blood cell counts before the onset of treatment ($12,820 \pm 580$ cells/mm³ in SO animals, $12,300 \pm 365$ cells/mm³ in the MI group and $12,258 \pm 398$ cells/mm³ in the MI-GCSF group, $P > 0.05$). Immediately before the surgical procedure, there was no difference between SO ($13,272 \pm 413$ cells/mm³) and MI ($12,535 \pm 320$ cells/mm³) rats. However, a significant increase in white blood cells was observed in the G-CSF pre-treated group ($22,673 \pm 849$ cells/mm³) was designated to undergo the coronary occlusion.

Proteins expression and apoptotic nuclei detection

To evaluate the effect of G-CSF on the intrinsic apoptotic pathway, protein expression was evaluated twenty-four hours after the final dose of G-CSF or saline in pre-treated rats (the animals were killed at the time that they would have undergone the surgery). As observed in Fig. 1, Bcl-2 (0.88 ± 0.09 in G-CSF vs. 0.52 ± 0.06 in control, $P < 0.05$) and Bcl-xL (0.56 ± 0.08 in G-CSF vs. 0.3 ± 0.02 in control, $P < 0.05$) protein expression increased significantly in the LV of rats subjected to G-CSF pre-treatment, suggesting that G-CSF treatment enhances protection from apoptosis in the myocardium. However,

Fig. 1. Left ventricle protein expression of Bcl-2 (26 kDa), Bcl-xL (30 kDa) and Bax (23 kDa) by Western blot, immediately before myocardial infarction. Control (n=6) and G-CSF (n=6). Data are mean \pm SEM. * $P < 0.05$.

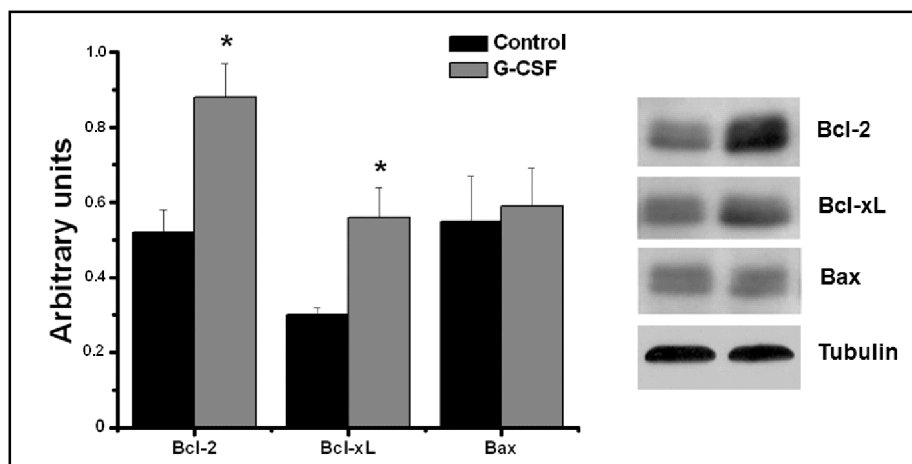
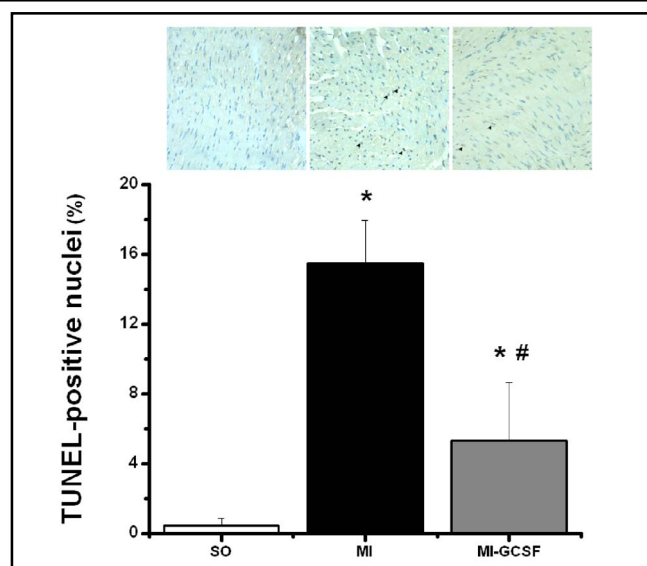


Fig. 2. TUNEL-positive nuclei evaluated in the LV twenty four hours after MI or fictitious surgery. Head arrow shows apoptotic nuclei. Data were compared using Kruskal-Wallis test and are depicted as mean \pm SEM. * $P < 0.05$ vs. SO; # $P < 0.05$ vs. MI.

protein expression of Bax remained unchanged with G-CSF pre-treatment (0.59 ± 0.1 in G-CSF vs. 0.55 ± 0.12 in control, $P > 0.05$) (Fig. 1).

Twenty-four hours after myocardial infarction, apoptotic nuclei were evaluated using a TUNEL assay. Fig. 2 shows an increase in TUNEL-positive nuclei after MI, which was reduced by G-CSF pre-treatment (SO: 0.46 ± 0.42 , MI: 15.5 ± 2.43 , MI-G-CSF: $5.34 \pm 3.34\%$; $P < 0.05$).



Myocardial infarction and chamber weight

Twenty-four hours after coronary occlusion, TTC staining showed that pre-treatment with G-CSF reduced infarct area compared to the non-treated rats ($36 \pm 2.8\%$ vs. $44 \pm 2.1\%$ in the MI group; $P < 0.05$) (Fig. 3). Similarly, two weeks after coronary occlusion, the infarct size was also significantly reduced in MI-G-CSF rats compared to MI rats ($27.2 \pm 2.2\%$ vs. $35.9 \pm 1.4\%$ in the MI group, $P < 0.05$) (Fig. 4B). However, the scar weight was similar in both groups (0.33 ± 0.03 in MI and 0.27 ± 0.03 g in MI-G-CSF), suggesting that the scar thickness in G-CSF pre-treated animals was greater than in the untreated infarcted group. Right ventricle hypertrophy was not observed in MI group, despite a trend ($P = 0.07$).

Hemodynamic parameters and LV pressure-volume relationship

Table 1 shows the results of hemodynamic measurements 15 days after SO or coronary ligation. The cardiac catheterization showed an increase in

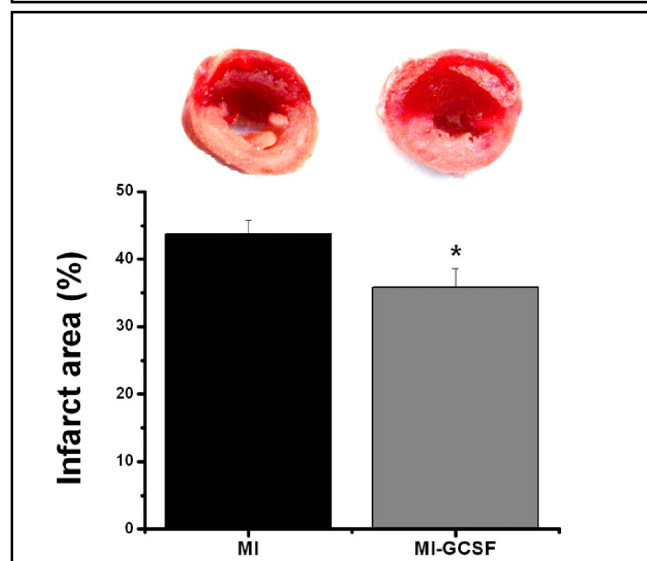
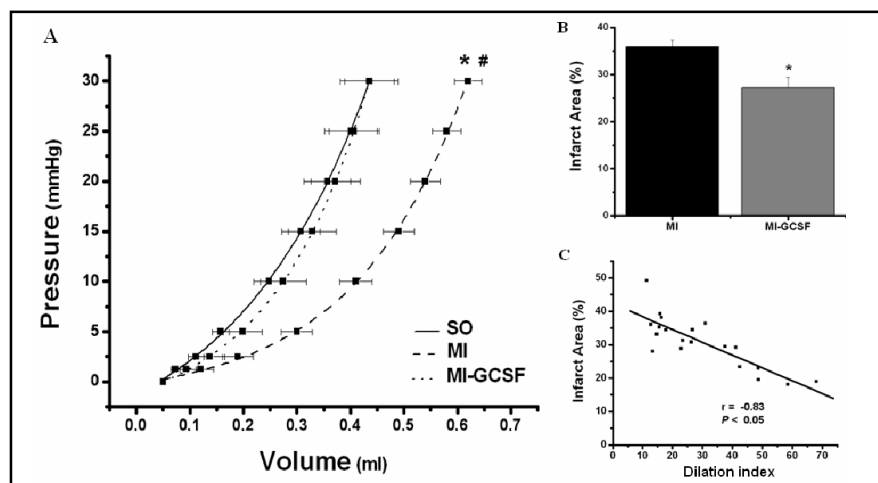


Fig. 3. Infarct size measured 24 hours after coronary occlusion, by TTC method, depicted as percentage of infarcted area to total LV area. MI (n=17) and MI-G-CSF (n=18). Data were compared using Student t test and are depicted as mean \pm SEM. * $P < 0.05$ vs. MI.

Fig. 4. (A) LV pressure-volume curve at 0 to 30 mmHg. (B) Infarct area evaluated two weeks after coronary occlusion. (C) Correlation between infarct area and dilation index. Data are mean \pm SEM. * $P < 0.05$ vs. SO; # $P < 0.05$ vs. MI-GCSF.



LVEDP and a decrease in peak LVSP and dP/dt after infarction. Animals infarcted after pre-treatment with G-CSF had a preserved LVSP (Table 1) and a reduced increase in LVEDP. Both LV contractility ($+dP/dt$) and relaxation ($-dP/dt$) were less affected in rats pre-treated with G-CSF.

Fig. 4 (panel A) shows the LV pressure-volume curves obtained in arrested hearts in all groups. As expected, myocardial infarction induced a rightward shift, indicating more intra-ventricular volume at the same distending pressure. However, G-CSF pre-treatment abolished this effect (SO: 42.8 ± 3.5 ; MI: 19.9 ± 2.5 ; MI-G-CSF: 41 ± 5 mmHg/mL; $P < 0.05$), indicating that G-CSF reduces LV dilation caused by myocardial infarction. A strong negative correlation ($r = -0.83$, $P < 0.05$; Fig. 4C) was observed between the LV dilation index and infarct size, suggesting an effect of infarct size on the LV dilation process.

To further evaluate the effect of changes in infarct size on the reduced left ventricular dilation found in G-CSF treated animals, we conducted a subgroup analysis (seven animals per group) by comparing the pressure-volume curves of MI and MI-G-CSF rats with paired infarct areas. LV pressure-volume relation was similar in both infarcted groups (MI = 21.2 ± 2 ; MI-G-CSF = 25.1 ± 4.2 ; $P > 0.05$, Fig. 5), suggesting that the reduction in LV dilation produced by G-CSF pre-treatment is related to the early reduction in infarct size.

Discussion

Our results show that pre-treatment with G-CSF preserves left ventricular function through early infarct size reduction and the attenuation of cardiac remodeling after MI, even after the treatment was stopped. These

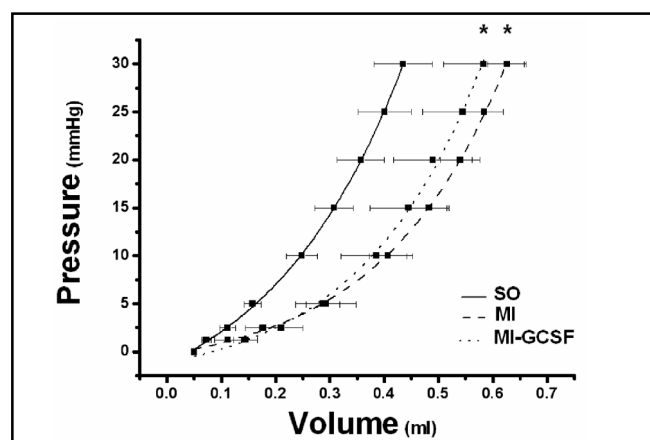


Fig. 5. LV pressure-volume curve performed in a sample of infarcted animals with same infarct area. * $P < 0.05$ vs. SO.

	SO (n=8)	MI (n=10)	MI-GCSF (n=11)
BW (g)	312 ± 6	320 ± 6	314 ± 8
LV/BW (mg/g)	1.95 ± 0.05	2.00 ± 0.04	2.01 ± 0.07
RV/BW (mg/g)	0.63 ± 0.05	0.72 ± 0.05	0.65 ± 0.03
HR (bpm)	244 ± 10	239 ± 11	263 ± 10
LVSP (mmHg)	115 ± 3	$102 \pm 2^*$	$111 \pm 2^\#$
LVEDP (mmHg)	8 ± 1	$18 \pm 4^*$	$12 \pm 3^\#$
$dP/dt+$ (mmHg/s)	4424 ± 141	$2886 \pm 294^*$	$3759 \pm 393^\#$
$dP/dt-$ (mmHg/s)	3227 ± 136	$2130 \pm 190^*$	$2714 \pm 205^\#$

Table 1. Hemodynamic and morphometric parameters at two weeks after myocardial infarction HR, heart rate; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end diastolic pressure, dP/dt , maximum rate of pressure rise and fall; BW, body weight; LV, left ventricle; RV, right ventricle. Data are presented as mean \pm SEM. * $P < 0.05$ vs. SO # $P < 0.05$ vs. MI.

results suggest that the long-term benefits found in G-CSF pre-treated rats are associated with anti-apoptotic protein upregulation and an early reduction in cardiomyocyte apoptosis. Moreover, our results strongly suggest that the reduced chamber dilation found in the

G-CSF pre-treated rats was dependent on the reduction in infarct size.

Mobilized bone marrow-derived stem cells can migrate to the ischemic heart to presumably help regenerate the infarcted myocardium, as previously reported [7, 16]. The homing of bone marrow-derived cells is mediated by stromal-derived factor (SDF-1) interaction with the receptor CXCR-4. These CXCR-4+ cells help mediate angiogenesis and cell survival in the myocardium [17]. SDF-1 is upregulated soon after myocardial infarction. As reported by Misao et al. [18], specific inhibition of the CXCR-4 receptor blunted the effect of G-CSF in infarcted rabbits. Moreover, it was reported that this pathway is also impaired by the action of the dipeptidylpeptidase IV, reducing cell homing and compromising the beneficial effects of G-CSF in the infarcted heart [19]. However, further studies have shown that the contribution of this regenerative process, if existent, is too modest to explain the improvement in cardiac function related to this cytokine [20]. More recent studies have demonstrated that post-infarction treatment with G-CSF improves LV function and survival, showing that this therapy may influence cardiac remodeling after myocardial infarction [12, 21, 22]. All of these studies assessed only the chronic effects of G-CSF, while its acute effects were not addressed.

Our results, in agreement with other studies, suggest a direct cytoprotective effect of G-CSF in the ischemic heart. For instance, the addition of G-CSF to an isolated perfused rat heart subjected to an ischemic injury led to an early reduction in the ischemic lesion and an improvement in LV function, showing a direct and likely non-genomic effect of G-CSF on the ischemic myocardium [23]. Harada et al. [24] failed to demonstrate a reduction in infarct size three days after occlusion; however, the authors showed a significant long-term improvement in cardiac function in G-CSF-treated mice. Here, we show that G-CSF pre-treated animals have increased expression of myocardial anti-apoptotic proteins such as Bcl-2 and Bcl-xL. Therefore, G-CSF-treated animals, being protected from early ischemic insult, were subjected to coronary ligation. Pre-treatment with G-CSF reduced infarct size twenty-four hours after coronary occlusion. This reduction was associated with a significant reduction in cardiomyocyte apoptosis in the ischemic area. Similarly, Harada et al. [24] observed increased levels of Bcl-2 and Bcl-xL in the myocardium twenty-four hours after infarction, but they did not observe a reduction in infarct size until seven days later. Another study showed that G-CSF-mobilized cells have enhanced expression of

cytoprotective genes that promote myocyte preservation and cardiac repair [25]. Apoptosis is an important kind of cell death in the early phase of myocardial infarction, contributing to the limitation of infarct size [3, 26, 27]. The increased levels of antiapoptotic factors observed in our study and the reduction in apoptosis soon after myocardial infarction may explain the reduced infarct size observed twenty-four hours after coronary ligation, which was maintained for two weeks following surgery.

In our study, even though pre-treatment with G-CSF was restricted to the period before coronary occlusion, infarct size also decreased in treated animals two weeks after infarction, which relates to the observed reduction in ischemic injury twenty-four hours after surgery. Therefore, this early reduction in infarct size may lead to fewer remodeling effects, preventing infarct expansion and directly impacting ventricular function. As reported, G-CSF improves left ventricular function in infarcted rats [12, 21, 24]. We found a similar effect two weeks after myocardial infarction, even without treatment following the surgery. This result suggests that the early effects of G-CSF pre-treatment account for this hemodynamic preservation. Accordingly, a significant infiltration of inflammatory cells into the ischemic tissue was detected two days after infarction in G-CSF treated rabbits, contributing to infarct repair and improvements in cardiac function [8]. Moreover, G-CSF expedites infarct repair through increased collagen deposition in the ischemic border zone, decreasing the left ventricular expansion [12]. Because our treatment was administered prior to myocardial infarction, the effects of this cytokine on the remodeling process are restricted to infarct size limitation through decreased apoptosis. Thus, improvement in cardiac function is related to changes in infarct size.

Pressure-volume curves showed that myocardial infarction contributed to left ventricular dilation as seen by the rightward shift of the pressure volume curve. Myocardial infarction leads to progressive left ventricular expansion, contributing to systolic dysfunction and heart failure [4]. We observed that pretreatment with G-CSF inhibits ventricular expansion and that pre-treated rats have a significant reduction in LV volume. Deindl et al. [28] showed that G-CSF treatment improved cardiac function and shifted the LV pressure volume-curve to the left. Moreover, chamber dilation correlates with infarct size, impacting ventricular function and survival [5, 29]. It is widely known that large infarct size is associated with a poor prognosis. Here, we observed a strong negative correlation between left ventricular dilation and infarct size, suggesting that the effects of G-CSF during

the early phase of myocardial infarction are essential in maintaining LV structure and function. To corroborate this conclusion, we analyzed a sample with similar infarct areas, and any differences were noted, underlining the crucial role of infarct size in determining post-infarction outcomes.

Taken together, our data indicate that the reduction in infarct size caused by the antiapoptotic effect of pretreatment with G-CSF is essential for its effects on post-infarction left ventricular remodeling. We cannot exclude the possibility that the observed effects of treatment with G-CSF are not just restricted to the acute phase. It has been previously reported that treatment with this cytokine in animals with chronic heart failure was sufficient to improve left ventricular function [30, 31]. Here, we show the important contribution of the acute effects of G-CSF in limiting heart failure progression. Therefore, the acute effects of G-CSF closely following infarction may help preserve long-term

cardiac function [32].

A clear limitation of our study is that G-CSF was administered before myocardial infarction, which is uncommon in clinical cases. However, our study clearly shows that after G-CSF pre-treatment, apoptosis is prevented in the early stages of myocardial injury, infarct size is reduced by up to 20% and LV dilation is limited, thus preserving LV systolic and diastolic function. However, the kinetics and dose response of G-CSF must be determined in further studies to attain optimal benefits.

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