Granulocyte Colony-stimulating Factor Reduces Mortality by Suppressing Ventricular Arrhythmias in Acute Phase of Myocardial Infarction in Rats

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Abstract: Our aim was to evaluate the effects of granulocyte colony-stimulating factor (G-CSF) on early cardiac arrhythmias after myocardial infarction (MI) and the impact on survival. Male Wistar rats received repeated doses of 50 μg/kg G-CSF (MI-GCSF group) or vehicle (MI group) at 7, 3, and 1 days before surgery. MI was induced by permanent occlusion of left coronary artery. The electrocardiogram was obtained before occlusion and then for 30 minutes after surgery. Events and duration of ventricular arrhythmias were analyzed. The levels of connexin43 (Cx43) were measured by Western blot immediately before MI production. Survival was significantly increased in MI-GCSF pretreated group (74% versus 52.9% MI, $P < 0.05$). G-CSF pretreatment also significantly reduced the ventricular premature beats when compared with the untreated-MI group (201 ± 47 versus 679 ± 117, $P < 0.05$). The number and the duration of ventricular tachycardia were smaller in the MI-G-CSF group, as well as the number of ventricular fibrillation episodes (10% versus 69% in MI, $P < 0.05$). Cx43 levels were significantly increased by G-CSF treatment (1.27 ± 0.13 versus 0.86 ± 0.11; $P < 0.05$). The MI size 24 hours after occlusion was reduced by G-CSF pretreatment ($36 \pm 3\%$ versus $44 \pm 2\%$ of left ventricle in MI group; $P < 0.05$). The increase of Cx43 expression in the heart may explain the reduced incidence in ventricular arrhythmias in the early phases after coronary artery occlusion in rats, thus increasing survival after MI.

Key Words: G-CSF, myocardial infarction, survival, ventricular arrhythmias

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INTRODUCTION

Sudden cardiac death (SCD) is the major complication of acute myocardial infarction (MI).[1] Ventricular arrhythmias are responsible for the majority of SCD,[2] and the occurrence of ventricular fibrillation was associated with poor survival in the first 30 days after acute MI.[3] Moreover, ventricular arrhythmias are highly associated with sudden death in infarcted patients within the first 6 months after coronary occlusion.[4]

Coronary artery ligation in rats has long been used as an experimental model to study either acute or chronic changes induced in the heart after myocardial ischemia.[5,6] Early mortality after experimental infarction has been described in some reports, and the main cause is related to cardiac arrhythmias.[7] Opitz et al[8] demonstrated that arrhythmias after coronary artery occlusion in rats can be divided into 2 distinct phases: an initial phase that includes the first 30 minutes after occlusion, and a second phase that includes the period between 90 minutes and 9 hours after infarction. It has been proposed that electrical uncoupling between myocytes plays a key role in delayed conduction and in generating an arrhythmogenic substrate in the acute phase of myocardial ischemia[9] as well as contributing to heart failure development in delayed phases of ventricular remodeling.[10] Increase of premature ventricular complexes has been shown in the cardiac-restricted connexin43 (Cx43) mice as compared to control animals.[11] Accordingly, the reduced Cx43 expression is associated with increased incidence of ventricular tachyarrhythmias after coronary artery occlusion in mice.[12] It was recently shown that transplantation of embryonic cardiomyocytes to infarcted mice reduced the incidence of VT in presence of a high degree of electrical coupling of the transplanted cells with the host myocardium.[13] Therefore, the degree of myocyte electrical uncoupling seems to exert a critical role for arrhytmogenesis in the ischemic myocardium.

Granulocyte colony-stimulating factor (G-CSF) is a hematopoietic cytokine that stimulates granulocyte proliferation and also mobilizes bone marrow stem cells.[14] It has been demonstrated that pretreatment of mice with G-CSF and stem cell factor (SCF) improves cardiac function and determines a great reduction in post-infarction mortality.[15] More recently, Ohtsuka et al[16] observed that G-CSF was able to reduce mortality after MI independently of SCF use. Moreover, G-CSF/SCF treatment reduces inducible ventricular arrhythmias in the chronic phase of MI in mice, an effect related to the increase in Cx43 expression.[17] Kuwabara et al[18] have recently

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shown that G-CSF ameliorates cell-to-cell coupling by stabilization of gap junctions in cardiomyocytes submitted to hypoxic conditions, and the post-infarction tachyarrhythmias were decreased in G-CSF pretreated rats.[18] Our purpose in this work was to study the post-infarction mortality in rats pretreated with G-CSF and to establish a quantitative measurement of ventricular arrhythmogenesis in the early stages of ischemia after coronary occlusion.

METHODS

Groups and Treatment

Male Wistar rats (200 to 250 g) were randomly selected to receive repeated doses of vehicle (MI group) or G-CSF (50 μg/kg; sc) (MI-GCSF group) at 7, 3, and 1 days before the surgical procedure to produce MI. During the experimental procedures, animals had free access to water and standard rodent food, and the experiments were performed in accordance to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

MI and ECG Measurements

Rats were anesthetized with sodium pentobarbital (60 mg/kg, ip), and MI was produced as previously described.[6] Briefly, a left thoracotomy was performed at the fourth left intercostal space, and the heart was quickly exposed. The left coronary artery was permanently occluded with a 6.0 suture, the heart was returned to the initial position, and the thorax was closed. Sham-operated (SO) animals were submitted to all procedures, except the coronary artery occlusion.

Subgroups of coronary ligated and SO rats were selected to record the electrocardiographic changes after infarction. Metallic electrodes were inserted into the subcutaneous space of the fixed limbs in the animals in dorsal decubitus. The ECG was continuously recorded for 30 minutes after thorax closure through an analog-to-digital conversion data acquisition system (BioAmp, ADInstruments, Australia), displayed on a computer screen, and saved for offline analysis of the cardiac rhythm. Twenty-four hours after surgery, the animals were euthanized, and the heart was rapidly removed and sliced in 4 transversal slices. The infarction size was determined by incubating the left ventricle in 1% triphenyltetrazolium chloride (TTC) in buffer solution for 10 minutes at 37°C. Under microscopic visualization, a careful dissection of the infarcted region from the surviving muscle was performed. The infarcted and non-infarcted areas were measured by planimetry, and the results were expressed as percentage of infarcted area to the total left ventricular area.[6]

Arrhythmia Analysis

Some parameters of Lambeth Convention[19] were used to evaluate the incidence of ventricular arrhythmias in the immediate (30 minutes) post-infarction period. The following parameters were measured: the number of ventricular premature beats (VPB), total duration of ventricular tachycardia (VT) periods (characterized by four or more consecutive VPBs in presence of atrial-ventricular dissociation), and occurrence of at least 1 episode of ventricular fibrillation (VF). Spontaneous reversion of VF was observed in some animals. When necessary, mechanical cardioversion was tested.

The incidence and the duration of ventricular arrhythmias were measured only in the animals that survived during the 30 minutes of recording.

A previously validated arrhythmia score[20,21] was used to quantify the abnormal electrical activity in all animals, including those that died during the ECG recording period. A score of 0 was attributed to animals without any ventricular arrhythmia; 1 to animals with only VPBs and/or fewer than 10 seconds of VT periods; 2 to animals with 11 to 30 seconds in VT; 3 to animals with 31 to 90 seconds in VT; 4 to animals with 91 to 180 seconds in VT and/or fewer than 10 seconds of reversible VF; 5 to animals with more than 180 seconds in VT and/or more than 10 seconds of reversible VF; and 6 to animals with irreversible VF.

Western Blot

To determine left ventricular Cx43 protein expression, a random sample (n = 5 in each group) of rats under G-CSF (n = 5) or vehicle (n = 5) treatment was sacrificed immediately before coronary occlusion to verify the possible effects of G-CSF without the interference of ischemic injury. The heart was rapidly removed and rinsed in physiological salt solution. The left ventricle myocardium, including the interventricular septum, was homogenized in 10 mM Tris-HCl, SDS 1%, and 1 mM sodium metavanadate medium (pH 7.4, 96°C). Protein concentration in the homogenate was determined by the Lowry method. A sample of 75 μg was submitted to a SDS–polyacrilamine gel electrophoresis (SDS-PAGE/12 %) and then transferred onto a nitrocellulose membrane. The membrane was blocked with Tris-buffered solution (10 mM Tris, 100 mM NaCl, and 0.1% Tween 20) with 5% of nonfat milk for 12 hours and then incubated with primary rabbit polyclonal antibody anti-Cx43 (1:1500 dilution; Zymed Laboratories, CA) followed by horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:1000 dilution; Bio-Rad, Hercules, CA). The bands were identified with an enhanced chemiluminescence system (ECL; Amersham International, Little Chalfont, UK), and the optical densitometric analysis was assessed with Scion Image software (Scion Corporation, Frederick, MD). Results were expressed as Cx43 signal, normalized to α-tubulin (mouse anti- α-tubulin, 1:500 dilution; Zymed Laboratories, CA) in each sample.

Statistical Analysis

Differences between 2 groups were accessed by unpaired Student *t* test, and the heart rate (HR) was assessed by 2-way ANOVA. A Kaplan-Meier curve was obtained for post-infarction survival analysis, and differences between groups were assessed by the log-rank test. Incidence of ventricular fibrillation was compared by Fischer exact test, and chi-square test was used to analyze different segments of the Kaplan-Meier curve. Area under the curve (AUC) was used to represent the VPB distribution along the 30 minutes of ECG recording. The data are presented as mean ± standard error of mean (SEM), and statistical significance was fixed at $P < 0.05$.

RESULTS

Survival Analysis

A total of 174 animals were used in the different protocols of this study: 87 in the MI group, 73 in the MI-GCSF group, and 14 in the SO group. Figure 1 shows the pooled data relative to the Kaplan-Meier survival curves for these groups. Twenty-four hours survival after infarction was significantly increased by G-CSF pretreatment (74.0% in MI-GCSF group versus 52.9% in MI; $P < 0.05$). No death occurred in the SO group. Considering only the first 30 minutes after infarction, we observed that survival was also significantly increased in the MI-GCSF group (89% versus 72.4% in MI group, $P < 0.05$). However, evaluating mortality occurring in the interval between 90 minutes to 9 hours, no significant difference ($P = 0.24$) between the 2 infarcted groups was observed.

Analysis of Post-infarction Arrhythmias

Post-infarction arrhythmia evaluation was determined in subgroups of SO rats (n = 6), MI-GCSF rats (n = 10), and untreated MI rats (n = 16). The heart rate measured by P waves in intervals with regular sinusal rhythm did not change significantly in any group (data not shown).

Table 1 shows that MI group presented the greatest number of VPBs (679 ± 117) during the 30 minutes after coronary occlusion. In contrast, pretreatment with G-CSF resulted in a significant reduction in VPB frequency early after coronary occlusion (201 ± 47). In addition, events of VT were also significantly reduced in MI-GCSF (7 ± 2 versus 29 ± 6 in MI, $P < 0.05$). In the MI group, 11 of 16 rats developed VF episodes; in the MI-GCSF group, only 1 of 10 animals showed this arrhythmia along the 30 minutes of ECG monitoring ($P < 0.05$).

The time course of VPBs after coronary occlusion is depicted in Figure 2, and the AUC shows that GCSF pretreated rats have a reduced number of VPB events as compared with the untreated MI group (543 ± 47 versus 165 ± 23, $P < 0.05$) (Figure 2, upper panel).

TABLE 1. Post-infarction Arrhythmic Activity

	SO	MI	MI-GCSF
N	6	12	9
VPB (events)	–	679 ± 117	201 ± 47*
Ventricular tachycardia			
Events	–	29 ± 6	7 ± 2*
Duration (seconds)	–	43 ± 9	13 ± 4*
VF (% incidence)	–	69	10*

*$P < 0.05$; VPB, ventricular premature beat; VF, ventricular fibrillation.

Considering the data obtained in the animals used for the arrhythmia analysis and considering the scores described in Methods, we observed a significant reduction in the arrhythmia score in the MI-GCSF group as compared to the untreated MI group (2.1 ± 0.5 versus 4.1 ± 0.4; $P < 0.05$).

Cx43 Protein Expression

The left ventricular Cx43 protein levels are shown in Figure 3. We observed a significant increase of Cx43 expression in rats subjected to G-CSF pretreatment as compared in control (1.27 ± 0.13 versus 0.86 ± 0.11 Arbitrary Units; $P < 0.05$).

Infarct Size

The MI size measured by TTC method 24 hours after coronary occlusion showed that G-CSF pretreated animals showed an infarct size smaller as compared with MI group (36 ± 3% versus 44 ± 2%, $P < 0.05$) (Figure 4).

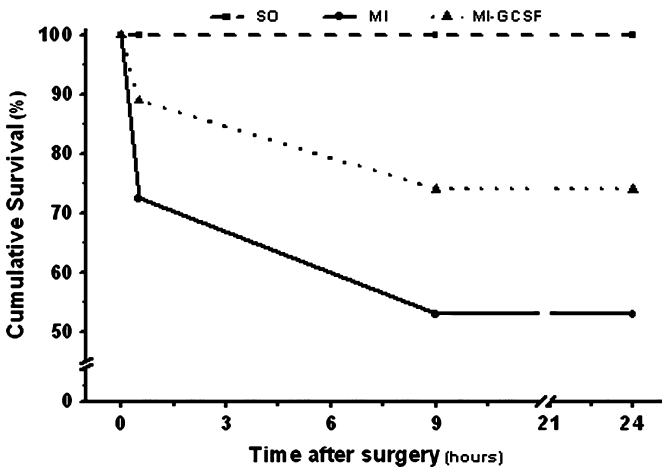

FIGURE 1. Kaplan-Meier curve of survival after sham operation (SO) and myocardial infarction without (MI) or with pretreatment with G-CSF (MI-GCSF). Each curve indicates the percentage of animals that died or survived after coronary ligation. Observe that the main effect of G-CSF treatment was observed 30 minutes after coronary occlusion.

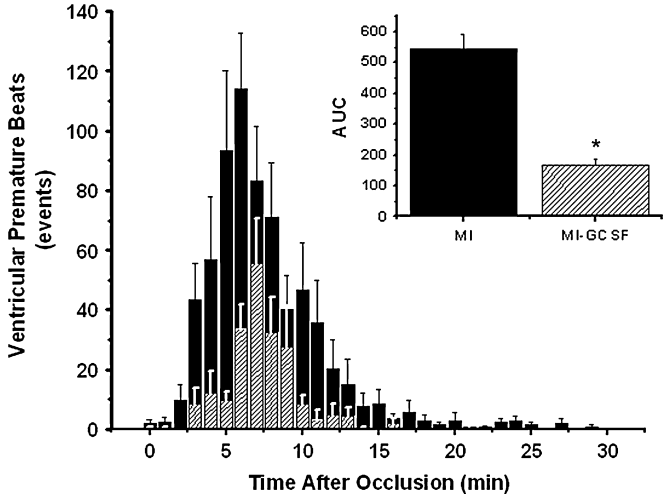

FIGURE 2. Time course of ventricular premature beats after myocardial infarction. Closed bars represent rats without treatment (n = 12), and open bars represent the rats pretreated with G-CSF (n = 9). The inset shows the area under the curves for both groups. Data are presented as mean ± SEM. *$P < 0.05$ versus MI.

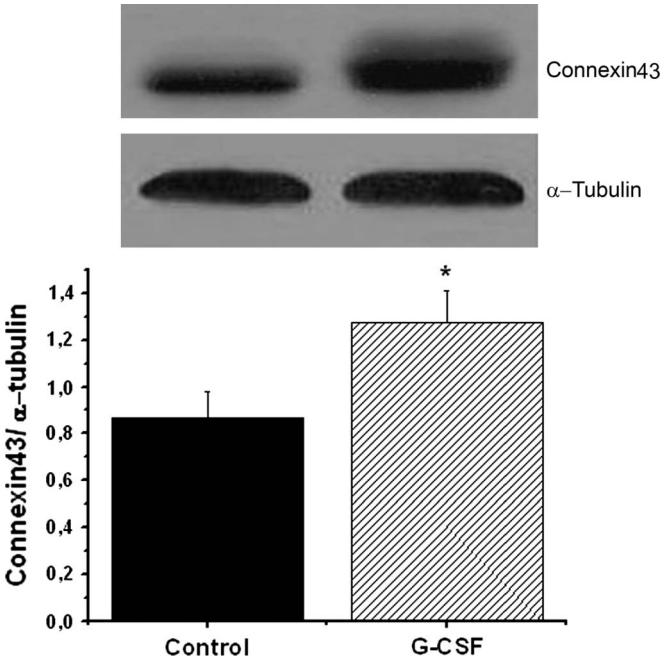

FIGURE 3. Connexin43 protein expression in left ventricle of rats in Control (n = 5) and G-CSF (n = 5) groups immediately before coronary occlusion. Results were expressed as ratio between signal for the connexin43 and signal for α-tubulin. Data are mean ± SEM. *$P < 0.05$ versus Control.

DISCUSSION

Previous studies have shown that treatment with G-CSF alone or in combination with SCF improves long-term post-infarction survival in mice,[15,16,22] and these results were related to remodeling attenuation and heart failure prevention. Our results show that long-term survival in infarcted rats submitted to previous treatment with G-CSF was mostly dependent on the reduced mortality observed in these animals during the first 24 hours after coronary occlusion. Mortality within this period is highly dependent on infarct size and on development of cardiac arrhythmias. Presence of arrhythmias has been associated with poor prognostic and high mortality rate in humans during the acute and sub-acute phases of MI.[2,23] In animals, early mortality was related to the incidence of lethal ventricular arrhythmias.[7,24] On the basis of information that arrhythmogenic activity after coronary occlusion was divided into 2 distinct phases,[5,8] we have performed a time-specific analysis of the post-infarction mortality. We observed that the different mortality rates observed in untreated infarcted rats and in rats subjected to G-CSF pretreatment was fully dependent on the different deaths observed during the first 30 minutes after coronary ligation.

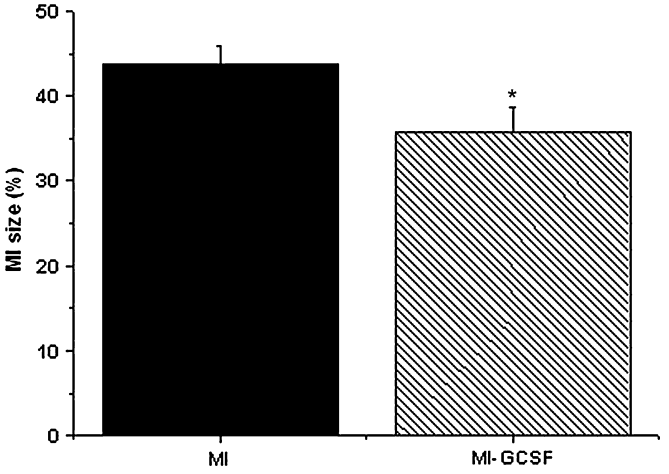

FIGURE 4. Myocardial infarction size 24 hours after coronary occlusion, expressed as percentage of infarcted area to left ventricular total area. MI (n = 17) and MI-G-CSF (n = 18). Data are mean ± SEM. *$P < 0.05$ versus MI.

Early electrical changes caused by acute ischemia lead to delayed conduction and create a specific substrate for the development of ectopic activity in the ischemic border zone facilitating development of VT and VF.[25,26] In accordance with the survival curve generated in our study and the pattern of arrhythmogenic activity in the post-infarction period previously described in other studies,[5,8,24] we observed significant reduction in the arrhythmogenesis in the G-CSF treated rats. We observed that nontreated infarcted animals showed a great incidence of VPBs, as previously demonstrated.[5,24] G-CSF pretreatment was effective in reducing the incidence of these events during the early phase after coronary ligation These data are in agreement with the results of Kuwabara et al,[18] who also recently observed reduction of VT and VF in rats with coronary artery ligature pretreated with a single dose of G-CSF 24 hours before coronary ligation. Cultured myocytes exposed to G-CSF showed increased expression of proteins involved in intercellular connection. In order to further explore a possible antiarrhythmic effect of G-CSF pretreatment, we decided to use a quantitative method to evaluate the severity of ventricular arrhythmias by using a validated score system.[20,21] This approach is effective because it allows inclusion in the analysis of all animals studied, including those that died during the observation period. We observed a reduction in the arrhythmia score in rats that received G-CSF pretreatment. These results strongly suggest that the improvement in early survival rate observed after infarction in G-CSF–pretreated rats may depend on the prevention of lethal ventricular tachyarrhythmia development.

It has been shown that G-CSF plus SCF reduces the incidence of inducible ventricular tachycardias in healed infarcted hearts by enhancing Cx43 expression and thus hinder the slowing in conduction in the infarction border zone.[17] This conduction delay caused by electrical uncoupling of cell layers at the border zone of ischemic tissue is the major cause of reentrant ventricular arrhythmias.[27] It has been shown that mice with reduced Cx43 expression have slowed conduction that induces an arrhythmic susceptibility.[28] Moreover, mice with cardiac-restricted Cx43 expression despite presenting normal cardiac structure die due to ventricular arrhythmias at approximately 2 months of age, suggesting an important participation of Cx43 in the development of sudden cardiac death.[29] In dogs[30] and pigs[31] submitted to infarction, this early arrhythmogenic phase, called phase 1 arrhythmias, have 2 distinct periods, named 1a (2 to 10 minutes) and 1b (12 to

30 minutes). Some authors relate that electrical uncoupling mediated by Cx43 is involved in the development of 1b phase arrhythmias. In rats, however, these distinct phases were not observed,[5,8,24,32] and most of the tachyarrhythmias occurring after coronary ligation seem to be mostly due to changes of the resting membrane potential (delayed conduction) and intercellular uncoupling.[26] Our data show a significant increase of Cx43 levels in the ventricular muscle of rats treated with G-CSF when compared to untreated control animals. It is likely that this effect may represent a key factor responsible for the increase of the electrical stability of the ischemic myocardium, thus preventing tachyarrhytmia development and reducing mortality. It is noteworthy that, although G-CSF–pretreated animals showed increased Cx43 expression, the mortality after 30 minutes after coronary ligation was not altered by treatment, suggesting that the mechanisms responsible to reduce the phase 1 arrhythmogenesis do not necessarily have important influences on delayed phase (phase 2) ischemia-induced arrhythmias. Some studies tried to identificate the mechanisms involved in development of VF in phase 2,[33,34] but little evidence was achieved.

Deletion of the N-cadherin gene in mouse causes a decrease of conduction velocity in the heart leading to an arrhythmogenic state, showing that stabilized gap junctions are essential for electrical stability.[35] It has been previously described that G-CSF increases gap junction-associated protein levels, such as Cx43, β-cadherin, and β-cathenin, thus contributing to preserve the tissue electrical stability.[18]

We observed a reduced infarct size in rats with G-CSF pretreatment. Reports have shown that G-CSF pretreatment was able to reduce myocardial injury.[15,16,22,36] Other studies, however, failed to observe these beneficial G-CSF effects.[37,38] These data, however, were recorded at the subacute or at the chronic phase of MI, when the degree of ventricular remodeling represents a key factor to the cardiac adaptations after infarction. In our experiments, the infarct size was evaluated 24 hours after coronary ligation, suggesting that G-CSF pretreatment may reduce the myocardial damage produced by ischemia. This G-CSF cardioprotection may depend on its antiapoptotic action. Ohtsuka et al[16] showed reduced apoptotical cells at the ischemic border zone of G-CSF–pretreated rats. Moreover, Harada et al[39] described that activation of the G-CSF receptor in cardiac myocytes leads to downstream activation in Jak/Stat pathways and an increase in Bcl-2 and Bcl-xL expression. Another pathway involved in G-CSF effect is the phosphatidylinositol 3-kinase (PI3K)/Akt. In a canine model of ischemia/reperfusion, endovenous G-CSF infusion reduced MI size, and a PI3K inhibitor abolished this effect.[40] In isolated rat hearts, the ischemic damage was also reduced by G-CSF perfusion, and this effect was related to PI3K/Akt phosphorilation of eNOS.[41] Furthermore, G-CSF–pretreated mice showed an increase in capillary density at ischemic border zone and decrease of apoptotical vascular endothelial cells 14 days after infarction.[16] Moreover, increase in arteriogenesis was related to the reduction of MI in G-CSF–treated mice.[22]

We cannot rule out the possibility that the small decrease of the infarct size in G-CSF–treated rat may partially contribute to the increased survival in the first 24 hours after coronary ligation. However, our data strongly suggest that the attenuation of ventricular tachyarrhythmias may be the main cause of the increased survival in this group of animals. It is likely that the increased Cx43 expression may improve electrical cell coupling, thus reducing reentrant ventricular pathways involved in the appearance of these harmful arrhythmias in the ischemic myocardium, which give an important contribution to the early mortality after infarction.

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