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Stem Cells Transplantation in Myocardial Tissue Induces Pro-arrhythmic Effects and Promotes Reperfusion. Comparison between Intramyocardial and Intravenous Approach

Pennella S[1,4#], Reggiani Bonetti L[2#], Migaldi M[2], Farinetti A[3], Manenti A[3], Lonardi R[3], Giuliani E[2], Panini F[1] and Mattioli AV[1,4*]

[1]*Department of Life Science, University of Modena and RE, Italy*

[2]*Department of Diagnostic Medicine, University of Modena and RE, Italy*

[3]*Department of Surgery, University of Modena and RE, Italy*

[4]*Istituto nazionale per le Ricerche Cardiovascolari, Italy*

[#]*Equally contributed to the paper*

[*]**Corresponding author:** Anna Vittoria Mattioli, Department of Life Science, University of Modena and R.E, Via del pozzo, 71 41100 Modena, Italy, Tel: 39594224043; E-mail: annavittoria.mattioli@unimore.it

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Abstract

Background: The aim of study was to evaluate the histologic changes and pro-arrhythmic features of intramyocardial stem cells transplantation after myocardial ischemia.

Methods: In 21 New Zealand rabbits an ischemia/reperfusion injury was induced by temporary ligation of anterior coronary artery during cardiac surgical procedure. Mesenchymal bone marrow stem cells (BMSCs) were isolated, cultured and re-suspended for injection. BMSCs were injected at the peri-infarcted area and side effects were evaluated and correlated with histological changes. Ventricular premature contractions (VPCs) were recorded during surgery and after 7 and 21 days.

Results: Frequent VPCs were observed before and after cells administration. The 7th day after the surgery, the episodes of VPC are more frequent in the group that received i.m. BMSCs and in the group that received i.m. administration of saline, compared to animals treated with BMSCs i.v. (135 ± 23; 52 ± 12; and 38 ± 9, respectively, P <0.01 within groups), while, after 21 days, the number of VPC was higher in the group treated with BMSCs by i.m. compared to the group treated with BMSC i.v. compared to the group treated with saline i.m. (100 ± 35 vs. 58 ± 22;and 24 ± 12 i.m saline, P <0.001 within groups).

Conclusions: This study shows that the pro-arrhythmic effect shortly after surgical procedure is related to intramyocardial injection damage. Furthermore, the pro-arrhythmic effect developing later after surgery seems to be induced by a combination of immature BMSCs and intramyocardial administration.

Keywords: Arrhythmias; Bone marrow stem cells; Route of administration; Myocardial ischemia

Introduction

Stem cell therapy in the field of cardiovascular disease has gained a wide acceptance in the medical community [1]. Although there is enthusiasm for the possibility of repair ischemic heart with cells, mechanisms and side effects therapy remain controversial.

A great number of clinical trials on intramyocardial bone marrow cell injections in patients with chronic heart failure have shown improved regional left ventricular function; however, these improvements lead only to modest global functional changes [2,3].

Furthermore, there have been repeated concerns that intramyocardial delivery could trigger ventricular arrhythmias [4,5]. The major safety issue associated with cardiac cell therapy has been the occurrence of sustained ventricular arrhythmias in some patients injected with skeletal myoblasts [5]. It has been postulated that lack of functional gap junction could slow the electrical conduction and predispose to re-entry circuits [5]. Moreover, the therapeutic efficacy and the occurrence of arrhythmias could be affected by the cell delivery route. The intramyocardial (i.m.) route delivers cells selectively into target areas; however, the needle could induce mechanical injury and subsequent acute inflammation [6,7]. On contrary, the intravenous (i.v.) route can deliver donor cells more homogeneously with less mechanical damage and inflammation in the myocardium, but the rate of homing and engraftment is poor [7,8]. The aim of study was to evaluate the histological changes in myocardial tissue following intramyocardial injections of Bone marrow mesenchymal stem cells and the relationship with arrhythmias.

Materials and Methods

Experimental protocol – Induction of ischemia

In 21 New Zealand rabbits (POLISTAB, Unimore Modena, Italy) 11 males and 10 females, mean age 70.6 ± 6 days (range 60) and with mean weight 4.1 ± 0.5 kg (range 3.81-4.42), an ischemia/reperfusion

injury lasting 12 min was induced during cardiac surgical procedure [9]. The rabbits were sedated by intramuscular (quadriceps femoris) injection of 1-2 mg/kg^{-1} medetomidyne and 0.7-1 mg/kg^{-1} midazolam under the direct supervision of an animal handler in order to reduce stress reaction. An initial bolus of 2-3 mg/kg^{-1} of 1% propofol solution was administered 2 min prior to surgical positioning and every 5 min to maintain anesthesia. A muzzle mask was then positioned over the nose and mouth, and a bag-valve 0.5- 1 ventilation system was connected to an oxygen supply. Respiratory rate was monitored by counting reservoir volume variations. A 6-lead electrocardiogram (ECG) was recorded throughout the operation. A left thoracotomy was made through the fifth intercostals space and the pericardium was opened. To produce an anterior ischemia, a distal portion of the coronary was selected for ligation. The selected artery was temporarily occluded, and the extent of ischemia was visually and electrocardiographically assessed (ECG ELI 150, Mortara Rangoni Europe srl., Casalecchio di Reno, Bologna, Italy). A successful induction of ischemia was confirmed by elevation of the ST segment by more than 0.2 mV in leads I, II and aVL.

BMSCs collection and culture

BMSCs were aspirated from posterior iliac crest, were cultured and re-suspended in saline (NaCl 0.9%) for injection at the level of peri-infarcted zone (6 injections). BMSCs were isolated by magnetic separation (MACs Miltenyi Biotec srl., Calderara di Reno, Bologna, Italy).

Cells were cultured in DMED with 10% fetal bovine serum, penicillin G (100 U/mL) and streptomycin (100 U/mL) at 37°C in humid air with 5% CO_2. After 72 h, the cells that were adherent to the culture flask were maintained for propagation and non-attached cells were discarded by 4 changes of medium. BMSCs were cultured with DMED with 10 μmol/mL BrdU (Abcam, Biochemicals, Cambridge, UK) for 48 hrs in order to make the donor cells for further identification. Cultured donor cells were dissociated from the culture dishes with 0.25% trypsin (Life Technologies Europe BV, Monza, Italy), neutralized with culture medium, and collected by 2000 rpm centrifugation for 10 min at room temperature according to the manufacturer's instructions. Four passages have been performed. Injected therapy included 4x106 BMSCs (identified by superficial markers CD34, CD45, CD166) for a total of 0.2 mL. The nuclei of donor cells were labeled with BrdU 72 h before transplantation; 70% of nuclei were stained positive for BrdU in culture.

BMSCs treatment

The surgical delivery of the cells was performed with a specific syringe equipped with an angled 27 1/2 G needle. After the reopening of vessel, animals were randomly assigned to a group of treatment: Group 1 received i.m. injections of BMSCs; Group 2 received i.v. administration of BMSCs; and Group 3 received i.m. injection of saline (to evaluate pro-arrhythmic effect of i.m. injections). After delivery of the cells, a single chest tube was placed under direct visualization through a 5- mm site. The chest tube was removed after disconnection from positive pressure ventilation. The edges of the sternum were then brought together and fixed with Ethilon 5.0 sutures. The remaining surgical accesses were closed in two layers. The anesthetic was stopped; the animal was extubated when appropriate and allowed to recover. All animals survived after surgery and cell delivery was in good hemodynamic status. All animals received postoperative antimicrobial therapy (cephazoline 100 mg/kg i.m. twice/day for 3 days) and buprenorphine (0.3 mg i.m. twice/day for 3 days) for post-operative pain [9].

ECG monitoring

To evaluate the occurrence of arrhythmic event, an ECG 24-h recording system was localized over the neck of the animals and recordings were made at different time intervals: before surgery (time 0); one day after surgery (time 1); 7 days after surgery (time 2); 21 days after surgery (time 3). ECG was also recorded during all surgery time.

The hourly number of supraventricular (SVPC) and ventricular premature contractions (VPCs), episodes of sustained ventricular tachycardia (VT), ventricular fibrillation (VF), the morphological change over the follow up, and QT time were calculated and analyzed by using specific ECG analysis software. Electrocardiogram QT time and cycle length (RR interval) were measured manually using calipers. Leads II, aVL, and aVF were recorded before, during, and after the vessel occlusion at an ECG sweep of 25 mm/s and amplitude calibration at 10 mm/mV. Time intervals (QT and RR) were measured in at least five consecutive cardiac cycles and mean values were calculated. QT intervals were measured from the earliest onset of the QRS complex to the end of the T-wave. Using an intraindividual approach, the same lead was used consistently for all measurements. The point of T-wave offset was defined as the return of the T-wave to baseline. Baseline and occlusion measurements were performed in the same lead [10].

The animals were monitored for the next 21 days (time of sacrifice).

Ethical Issue

All animals received care in compliance with the European Convention on Animal Care. Animal care was provided by the same trained operator in order to reduce stress. The study was approved by the Research Animal Care and Use Committee of the University of Modena and Reggio Emilia (Italy) (prot. n. 25, 18/03/2008 and prot. n. 83, 25/09/2009).

Histology

After sacrifice, the infarcted zone was divided into 6 consecutive parts, numbered 1-6: part 1was in the zone nearest to the upper part of the heart and part 6 in that nearest to the apex. The 6 parts were: right and left ventricles, right and left atria, and interventricular and interatria septa. The heart of all rabbits was fixed in 10% buffered formaldehyde solution for 24 hours; 3 mm consecutive sections were performed starting from the apex to the heart base. Myocardial tissue was totally included in paraffin embedded blocks and sections of 7 μm of thickening were stained using the routinely hematoxylin and eosin.

All slides were examined by two pathologists and tissue features were described, in particular: presence of ischemic alterations, reperfused areas, presence of inflammatory cells, and other morphological alterations.

We defined as ischemic damage, the tissue with areas of myocardium showing vanishing myocytes nuclei, disappearance of typical transverse myocytes striations, and appearance of a homogeneous cytoplasm granulation, large pinocytosis cavities and wavy contraction bands. While, as sign of reperfusion, the tissue with areas of myocardium with normal myocytes, regularly distributed and intermingled to small vascular space lining by small endothelium and fulfilled by erythrocytes [11,12].

Statistical analysis

SPSS software, version 14.0.1 (SPSS Inc., Chicago, Ill, USA), was used for statistical analysis. Comparison of data between groups was performed by ANOVA. The t-test was used to compare data obtained by animals of the same group. P<0.05 was considered statistically significant. All data are expressed as mean ± SD.

Results

Pre BMSCs inoculation – ECG monitoring

Ventricular arrhythmias were observed in all animals during the different phases of the experimental protocol. One rabbit died before the ischemic induction for mycoplasma infection.

Of the 20 rabbits examined one developed VT and two animals experienced non sustained VT both during the opening of the pericardium and alternating phases of VT during the reperfusion phase.

Six rabbits showed pairs of VPC during the opening of the pericardium and the reopening of the vessel, one showed pairs of VPC during the opening of the pericardium, a bradycardia during the ischemia followed by a spontaneous recovery during reopening of the vessel.

Another animal developed pairs of VPC during the opening of the pericardium, a 1° AV block during ligation of the LAD and pairs and triplets of VPC during the reopening. This animal died 24 hours after surgery.

Nine cases developed triplets of VPC during the opening of the pericardium, one developed triplets of VPC during the opening of the pericardium and the ligation of the vessel, one developed triplets of VPC both during the opening of the pericardium, during ligation and reopening of the vessel.

The arrhythmic events according to the different phases of the experimental protocol were distributed as shown in the graph (Figure 1).

In detail, during the three phases of the experiment was recorded only one event of VT, while, were recorded 8 pairs of VPC (3 during the opening of the pericardium, 2 during the ligation of the vessel, and 3 during the reopening of the vessel) and 6 triplets of VPC (3 during the opening of the pericardium, 2 during the ligation of the vessel, and 1 during the reopening).

Post BMSCs inoculation – ECG monitoring

Of the initial group of 20 rabbits, 7 were treated with BMSCs i.m., 7 with BMSCs i.v. and 6 with NaCl 9% by i.m. in order to evaluate the effect of pro-arrhythmic injection therefore defined as the control. The main electrical parameters recorded in the three groups following the various treatments in different times are shown in Table 1.

	BMSCs Injection i.m.	BMSCs injection i.v.	NaCl injection i.m.
HR time 0	208 ± 22	206 ± 23	205 ± 20
HR during ischemia	185 ± 18*	198 ± 21*	186 ± 24*
HR time 1	212 ± 21	210 ± 20	208 ± 19
HR time 2	210 ± 19	207 ± 18	206 ± 15
HR time 3	209 ± 23	208 ± 20	203 ± 18
QTc interval time 0	420 ± 30	418 ± 36	416± 25
QTc interval time 1	431 ± 28**	309 ± 26**	436 ± 25**
QTc interval time 2	428 ± 19*	388 ± 28*	421 ± 34
QTc interval time 3	426 ± 23	416 ± 29	424 ± 28

Table 1: Electrical parameters recorded in the days following the administration of BMSCs. The values in the table are the means of the number of events collected in 20 rabbits. HR: Heart Rate; QTc: Corrected (Interval QT corrected); T0 (before surgery); T1 (1 day after surgery); T2 (7 days after surgery); T3 (21 days after surgery); *P<0.05 vs T0 pre-ischemia, **P<0.001vs T0 pre-ischemia

Figure 1

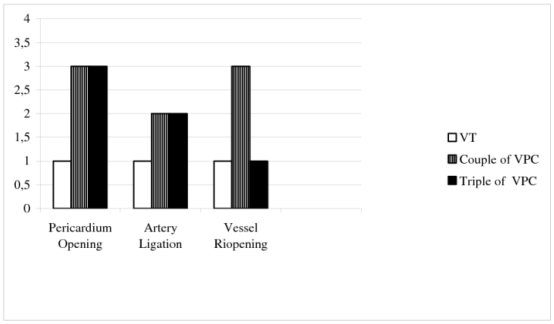

Figure 1: Distribution of arrhythmic events in 20 rabbits during the three phases of the experimental protocol: the opening of the pericardium, the LAD ligation and reopening of the vessel, prior to administration of BMSCs. VT: Ventricular Tachicardia; VPC: Ventricular Premature Contraction

Before (time 0) and after the surgery (time 1) significant arrhythmic events were not recorded. The 7th day after the surgery, the episodes of VPC are more frequent in the group that received the i.m. administration of BMSCs and in the group that received the i.m. administration of saline, compared to animals treated i.v. (135 ± 23; 52 ± 12; and 38 ± 9, respectively, P <0.01 within groups), while, after 21 days, the number of VPC was higher in the group treated with BMSCs by i.m. compared to the group treated with BMSC i.v. compared to the group treated with saline i.m. (100 ± 35 vs. 58 ± 22; and 24 ± 12 im saline, P <0.001 within groups) (Figure 2).

Figure 2

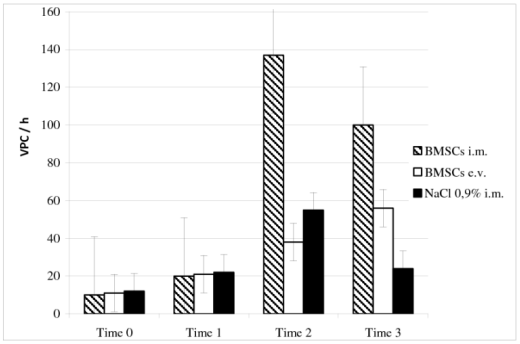

Figure 2: Number of premature contractions recorded in the three groups of animals correlated with the route of administration of BMSCs and in relation to different observation times. VPC: (Ventricular Premature Contraction); T0 (before surgery); T1 (1 day after surgery); T2 (7 days after surgery); T3 (21 days after surgery).

When we evaluate the occurrence of the pro arrhythmic event on the basis of treatment rather than on the route of administration, the number of VPC before and after the surgery appears comparable in the two groups and numerically of little significance, while the 7th and 21st day after surgery arrhythmic events are numerically higher in the group receiving the BMSCs compared to the group receiving placebo (Figure 3).

Figure 3

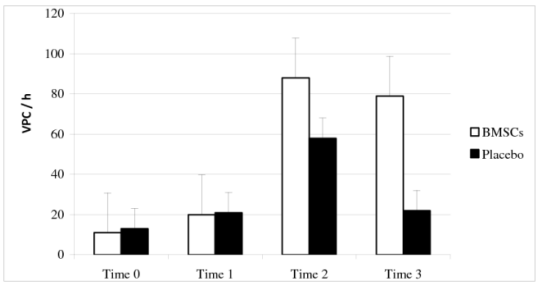

Figure 3: Number of premature contractions recorded in the three groups of animals correlated with the object of the administration in the different observation times. VPC: Ventricular Premature Contraction); T0 (before surgery); T1 (1 day after surgery); T2 (7 days after surgery); T3 (21 days after surgery)

The QT interval was increased in animals of all groups during the occlusion of the vessel, while in the group treated with i.m injection remained prolonged compared to the group treated with infusion intravenously After 21 days, the QT interval was prolonged in the group treated with i.m injection of BMSC from 412 ± 23 (pre-ischemia) to 443 ± 34 (day 7) up to 420 ± 28 (day 21).

Histological analysis

The examination showed normal morphology in 19 cases while a dampish left ventricular area in the remaining 1 case. In particular we observed in the majority of rabbits treated with BMSCs i.m. (6/7) and in only one case treated with BMSCs i.v., the presence of ischemic alterations and concomitant aspects of reperfusion.

The alterations of ischemic type were represented mainly by disappearing of the nuclei and the typical striations, pinocytosis cavitations and edema. While, the signs of reperfusion were represented by numerous small-size capillaries fulfilled by erythrocytes and the lack of wide necrosis area. Most of these rabbits also had a ripple myocardial (4/7).

A second group of rabbits, represented by all the cases treated with BMSCs i.v. (7/7) and some controls (2/6) showed characteristics of ischemic damage and less signs of reperfusion. In this, the signs of reperfusion were minimal and only minimally represented by few capillary filled with erythrocytes.

Finally, the remaining rabbits treated with NaCl 9% due to i.m. (4/6) showed ischemic alterations especially and bands of ripple (Figure 4).

Discussion

The present study reported the pro-arrhythmic complications of mesenchymal stem cells derived from bone marrow transplanted in a pre-clinical ischemia/reperfusion model. Nowadays, the efficacy of cell therapy in cardiovascular care is debated mainly because information on mechanisms involved into cardiomyocyte trans-differentiation is lacking [13-15]. Furthermore, some studies were prematurely interrupted by the occurrence of arrhythmia [4].

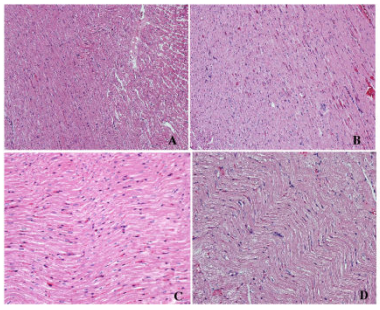

Figure 4

Figure 4: (A-B) Riperfused myocardial tissue with prominent thin and small vascular channel fulfilled by erythrocytes in post-ischemia background tissue. (C – D) Ripple myocardial in i.m. treated rabbit.

At the beginning the cell therapy used skeletal myoblasts derived from skeletal muscle satellite cells [16]. The choice of this type of cells was based on bioavailability, on the ability to proliferate and migrate

into ischemic areas. These cells do not differentiate in cardiomyocytes, but, they localize in cardiac muscle cells as mature skeletal muscle, although they were observed fusion events between cells. Moreover, these cells are not able to express the proteins of the gap junction and thereby to form electromechanical junctions with cardiomyocytes [17,18].

For this reason, the transplanted cells are functionally isolated from the adjacent myocardium and the transplanted area is functionally contractile and excitable, but not electrically coupled with cardiomyocytes. This lack of electrical coupling would explain the occurrence of the arrhythmic event [16].

The bone marrow includes a heterogeneous pool of cells represented mainly by hematopoietic stem and mesenchymal stem cells, and by a large number of undifferentiated progenitor cells.

Unlike skeletal cells that have been tested in chronic ischemic heart disease, the bone marrow cells were mostly administered in acute myocardial infarction. Despite stem cells derived from bone marrow are able to express the gap-junction, trans-differentiate into cardiomyocytes and to integrate with host myocardium, the results obtained on the arrhythmic event onset were discordant.

Pack et al. administering mesenchymal stem cells (MSCs) have highlighted a pro-arrhythmic status after cell transplantation related to proliferation with uneven distribution of the same in correspondence of the infarcted area [19]. Amado et al. have not observed arrhythmic events after i.m. injection of MSCs [20]. In addition, the pro-arrhythmic risk is not entirely related to the cells themselves but can be influenced by environmental factors mainly after an ischemic event. Ischemia is pro-arrhythmic "per se" and induced neurohumoral changes both systemic and local i.e.: release of cytokines, platelet activation, endothelial activation [1,13-15].

Based on these data, the aim of our study was to evaluate the occurrence of arrhythmia related with cell type (BMSCs), and the route of cell therapy administration.

We observed that none of animals developed significant arrhythmic events the first day after surgery, suggesting a common increased electrical instability with respect to baseline.

Seven days after surgery, the number of ventricular events increased significantly in the group treated with BMSCs i.m. and in the group treated with placebo i.m. compared to the group treated with BMSCs by i.v. This suggests that there was probably a pro-arrhythmic effect linked to intramyocardial injection.

After 21 days of transplantation, the number of arrhythmic events was reduced in the group treated with BMSCs i.m. and in the group treated with placebo i.m., suggesting that the damage caused by the injection, probably responsible for the pro-arrhythmic effect in acute phase, and then gradually come down.

Differently, the number of arrhythmic events observed in the group treated with BMSCs by i.v. had delayed increases, indicating a potential arrhythmic event that we hypothesized induced by a partial differentiation of cells in cardiomyocyte sense and probably by a partial integration of the same with the underlying myocardium. Homing was poor in patients treated with i.v. administration probably due to the low number of cells according to a previous study [10]. Therefore there was a pro-arrhythmic effect due to BMSCs which manifests itself both in the group treated i.m. that in the group treated systemically. In the first one, the occurrence of pro-arrhythmic event can be related to a lack of integration of the cells with the underlying myocardium, or, to a partial differentiation of the same. In the second one, instead, the smaller number of pro-arrhythmic events recorded could be related to the fact that not all the cells administered i.v. reach to ischemic site. We suppose that the cause of this poor homing could be related to the low number of cells administered and to a possible recruitment by the spleen or liver [21,22].

The administration of BMSCs i.m. determined a larger number of pro-arrhythmic events compared to administration of the cells by systemic and compared to placebo i.m. Certainly the pro-arrhythmic effect is induced also by the route which the cells are inoculated. The occurrence of the pro-arrhythmic event after i.m. administration would seem to be due to the effect induced by the i.m administration route and this method of approach appears to be the most harmful. In addition, the administration on the spot, also determines an amplification of the inflammatory process, already present because of ischemic injury [22].

The ECG analysis has shown arrhythmic events during the three phases of surgery (opening of the pericardium, coronary ligation, the reopening of the vessel) and consequently not correlated to cell therapy. In detail there has been only one event of VT, whereas, there have been 8 pairs of VPC (3 during the opening of the pericardium, 2 during ligation of the vessel and 3 during the reopening of the vessel) and 6 triplets of VPC (3 during the opening of the pericardium, 2 during the ligation of the vessel, and 1 during the reopening). These data suggest that surgery technique and post vessel-ligation ischemic damage provide a substrate pro-arrhythmic. Especially, the VPC events observed during the opening of the pericardium fall into the complications of the experimental model [9].

Arrhythmias recorded during ligation and the reopening of the vessel were similar to that occur in humans in clinical practice. As well known, our data confirm, that the ischemic event induces arrhythmias also in rabbit, an animal that usually not manifest frequent ventricular events [10].

An interesting data is the presence of new small vessels in the damaged myocardium closed to needle puncture, and we hypothesized that the new angiogenesis could be related to reperfusion. In these areas nuclear pycnosis was less and myocytes without nucleus were found. It is possible that both ischemic injury and stem cells promote angiogenesis [23]. Another peculiar histological feature observed in i.m. treated myocardium was the presence of "myocardial ripple". Because these ripples were absent in rabbit treated with i.v. route but were usually represented in controls in which the placebo was inoculated i.m, we consider they correlated with the procedure and not with the possible hyper-contractile action of the cells.

Limitation of the study: The major limitation of the study was related to the interval time between ischemia and delivery of cells. The administration of cell was performed during open heart surgery immediately after ischemia; this induced a bias related to the pro-arrhythmia effects induced by the ischemia and by the surgery. During the acute phase of the protocol we were not able to recognize arrhythmias induced by ischemia and/or surgery from arrhythmias induced by cells.

Conclusion

I.m. injections of BMSCs induced an electrical instability, as shown by a high number of VPCs and a longer QTc time, as compared with

i.m. injections of saline and with i.v. administration of BMSCs. The pro-arrhythmic status was related to the needle injury during injections and to the electrical remodeling induced by BMScs administration. Ventricular events have not yet determined the exitus of animals, if not in a single case before the administration of cell therapy.

These preliminary data need to be confirmed by further studies evaluating a longer follow up before translating information into clinical studies.

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