



HAL
open science

Autologous myoblast transplantation after myocardial infarction increases the inducibility of ventricular arrhythmias

Sarah Fernandes, Jean-Christophe Amirault, Gilles Lande, Jean-Michel Nguyen, Virginie Forest, Olivier Bignolais, Guillaume Lamirault, Didier Heudes, Jean-Luc Orsonneau, Marie-Françoise Heymann, et al.

► To cite this version:

Sarah Fernandes, Jean-Christophe Amirault, Gilles Lande, Jean-Michel Nguyen, Virginie Forest, et al.. Autologous myoblast transplantation after myocardial infarction increases the inducibility of ventricular arrhythmias. *Cardiovascular Research*, 2006, 69 (2), pp.348 - 358. <10.1016/j.cardiores.2005.10.003>. <inserm-01707095>

HAL Id: inserm-01707095

<https://inserm.hal.science/inserm-01707095v1>

Submitted on 12 Feb 2018

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

**Autologous Myoblast Transplantation after Myocardial Infarction Increases the
Inducibility of Ventricular Arrhythmias**

Sarah Fernandes, Pharm D; Jean-Christophe Amirault, MD; Gilles Lande, MD; Jean-Michel Nguyen, MD, PhD; Virginie Forest, PhD; Olivier Bignolais; Guillaume Lamirault, MD; Didier Heudes, MD, PhD; Jean-Luc Orsonneau, MD; Marie-Françoise Heymann, MD; Flavien Charpentier, PhD; Patricia Lemarchand MD, PhD.

Fernandes: Cardiac cell therapy and arrhythmias

From L'Institut du Thorax INSERM U533, Faculté de Médecine, Nantes; PIMESP, CHU Nantes; INSERM U652, Paris; Service de Biochimie, CHU Nantes; Service d'Anatomopathologie, CHU Nantes; Nantes, France

Corresponding author: Patricia Lemarchand, MD, PhD, L'Institut du Thorax INSERM U533, Faculté de Médecine, 1 rue Gaston Veil, F-44035 Nantes cedex 1, France

Tel: (33) 2 40 41 29 91 Fax: (33) 2 40 41 29 50

patricia.lemarchand@nantes.inserm.fr

Total word count: 5784

Introduction

Transplantation of myoblasts, the precursors of skeletal muscle fibers, has drawn worldwide attention as a promising alternative strategy to treat end-stage heart failure [1]. The basic assumption for such strategy is that left ventricular dysfunction is primarily caused by the loss of a critical number of cardiomyocytes, and that their replacement by new contractile cells could functionally “regenerate” postinfarction scars. Small scale clinical trials suggested the feasibility of myoblast transplantation into the injured myocardium and confirmed the improvement of global and regional left ventricular function, late after myocardial infarction [2]. However, symptomatic ventricular arrhythmias occurred with an unexpected frequency within weeks following cell transfer. In the first phase I trial of autologous myoblast transplantation, 4 out of 10 patients had episodes of sustained ventricular tachycardia, requiring the implantation of Implantable Cardiac Defibrillator (ICD) [3]. In another phase I trial, again a high frequency of both sustained and non-sustained ventricular tachycardias were encountered, leading to ICD implantation [4]. Such cardiac ventricular arrhythmias may be a serious limitation to myoblast therapy [2], and myoblast transplantation trials are now restricted to patients equipped with ICD. Investigators have hypothesized that the transplanted skeletal myoblasts remain committed to their lineage and retain an action potential duration different from that of adjacent myocardium. This electrical inhomogeneity may predispose to ventricular arrhythmias [5]. However, the relationship between transplantation procedure and ventricular arrhythmias may be confounded by the trend for patients with ischemic cardiomyopathies to develop arrhythmic events [6]. Alternatively, these arrhythmias could have an inflammatory origin due to both needle punctures and early cell death from necrosis and/or apoptosis, as shown in other models of cell transplantation [7,8]. Therefore, experimental *in vivo* evaluation of cell engraftment in animal models is highly desirable to

evaluate cardiac hyperexcitability after myoblast transplantation. Because ventricular arrhythmias are life-threatening diseases, such preclinical evaluation should also be important for any new source of cells to be implanted into the myocardium.

METHODS

All animal experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Myocardial Infarction

Male Wistar rats (200 g) were anesthetized with isofluran (3%) and tracheally ventilated (Harvard Rodent Ventilator, Harvard Apparatus). After left lateral thoracotomy, myocardial infarction was induced by ligation of the main branch of the left marginal coronary artery, 2 mm from the tip of the left auricle. Post-operative analgesia was obtained with nalbuphine (3 µg/kg s.c.; Nubain[®], SERB). Cardiac troponin I was measured 4 hours after coronary ligation, in a blood sample using a cardiospecific assay (AccuTnI[™] Access[®] immunoassay, Beckman-Coulter).

Cell Isolation

Isolation and Expansion of Autologous Skeletal Myoblasts

Forty-eight hours before skeletal muscle biopsy, 0.5 mL bupivacaine (Marcaine[®], Astra) was injected into the tibialis anterior muscle of rat hindlimbs. At day 0, immediately after coronary ligation the right and left tibialis anterior muscles were dissected and cell culture was performed as described by Pouzet *et al.* [9]. Briefly, muscles were minced and digested with collagenase IA (2 mg/mL, Sigma Chemical), and trypsin-EDTA (0.25%, GIBCO BRL) to release satellite cells. After filtration and centrifugation, cells were resuspended in the culture medium composed of F12 with 20% FBS (vol/vol), 1% (vol/vol) penicillin-streptomycin (10000 UI/mL–10 mg/mL, GIBCO BRL), 5 ng/mL basic fibroblast growth factor (Sigma Chemical) and dexamethason (10^{-6} mol/L). For autologous transplantation, on day 7 post-

coronary ligation cells were harvested by trypsinization, pelleted, and suspended in 150 μ L of F12 medium.

Autologous Bone Marrow Mononuclear Cells

On day 7 post-coronary ligation, autologous bone marrow was aspirated from the right femur and tibia with a 18 gauge needle. Bone marrow mononuclear cells (BM-MNC) were isolated by Ficoll gradient (Eurobio). A total of 15.10^6 autologous BM-MNC cells were suspended in 150 μ L of F12 medium.

Cell Transplantation

Myoblast or BM-MNC injections were performed, at day 7 post-coronary ligation, into the infarcted area via left thoracotomy under direct observation, using 3 intramyocardial injections of 50 μ L each with a 30 gauge needle. To identify transplanted cells within the recipient heart, cells were labeled immediately before cell injection using a green intracellular fluorescent dye, CFSE (Carboxy-Fluorescein diacetate Succinimidyl Ester, Molecular Probes) [10]. Cell labeling was verified by fluorescence microscopy before injection.

Electrophysiological studies

ECG recordings in conscious, free-moving rats

A telemetric transmitter (TA10EA-F20, Data Sciences International) was inserted into the abdominal cavity just before coronary ligation. The positive and the negative leads were tunneled to the left hindlimb and to the right shoulder, respectively. Telemetric recording of 24-hour periods started at day 2 after coronary ligation and were performed every 96 hours, up to 5 weeks. Data were analyzed by a blinded operator, using ECG Auto 1.5.7 software (EMKA Technologies). Ventricular premature beats were defined by the presence of at least

two out of three criteria: 1) atypical QRS configuration with alteration or inversion of the T wave, 2) post-extrasystolic pause, 3) atrioventricular dissociation.

Programmed electrical stimulation in sedated rats

Ventricular programmed electrical stimulation (PES) were performed in all groups by a blinded operator. An epicardial electrode (Electro Streamline, Medtronic) was tied to the viable left ventricular myocardium during surgery for coronary ligation and tunneled under the skin, allowing easy access. For PES stimulation, animals were sedated with a mixture of etomidate (8 mg/kg i.p.; Hypnomidate[®], Janssen-Cilag) and pentobarbital (40 mg/kg i.p.; Pentobarbital sodique[®], Cerva Santé Animale). The distal tip of the epicardial electrode was externalized to be used as the negative lead. Another electrode was placed on the ventral side of the thorax to be used as the positive lead, allowing unipolar stimulation (UHS 20, Biotronik). Surface six-lead ECGs were recorded using 25 gauge subcutaneous electrodes connected to a computer through an analog-digital converter (IOX 1.585, EMKA Technologies) for monitoring and later analyses. ECG channels were filtered out below 10 Hz and above 100 Hz. Analyses were performed with ECG Auto 1.5.7 software (EMKA Technologies). Standard criteria were used for interval measurements (RR, PR, QRS and QT). Pacing was performed by applying a 1-ms pulse pacing of width at 2 times higher than capture threshold. Standard clinical PES protocols were used, including single, double and triple extrastimuli applied under spontaneous rhythm or following a train of 9 stimuli at 100-ms drive cycle length. The coupling interval of the last extrastimulus was decreased by 2-ms steps from 80 ms down to the ventricular effective refractory period (VERP). Pacing protocols were interrupted if sustained ventricular tachycardia (VT) was induced. Sustained VT was defined as fast ventricular rhythm of 15 or more beats, according to Lambeth Conventions [11].

Tissue histology and morphometry

Sirius red staining was performed in paraffin embedded sections (3 μm). Histomorphometric analyses were performed using a computer-assisted image analyzer (NS 15000, Microvision Evry, France). Immunochemical staining was performed in serial cryosections (6 μm). A mouse monoclonal antibody against the fast skeletal myosin heavy chain (NCL-MHCf, Novocastra) and a rabbit monoclonal antibody against connexin43 (Zymed Laboratories) were used for identification of differentiated myotubes and connexin43, respectively.

Hypertrophy evaluation by TaqMan Real-time RT-PCR

Total RNA was isolated from each ventricular tissue sample with TRIzol Reagent (Life Technologies). RNA was DNase-treated (RNeasy Fibrous Tissue Mini Kit, QIAGEN).

First-strand cDNA was synthesized from 2 μg of total RNA using the High-Capacity cDNA Archive Kit (Applied Biosystems). On-line PCR was performed with the following primers: natriuretic peptide precursor type A (ANF, Rn00561661_m1), cardiac beta myosin heavy chain polypeptide 7 (β -MHC, Rn00568328_m1), and skeletal muscle alpha 1 actin (α -actin, Rn00570060_g1). Fluorescence signals were normalized to the hypoxanthine guanine phosphoribosyl transferase (HPRT, Rn01527838_g1), used as reference gene. All data were averaged and then used for the $2^{-\Delta\text{CT}}$ calculation. $2^{-\Delta\text{CT}}$ corresponds to the ratio of each gene expression *versus* HPRT [12].

Data Analyses

Data are expressed as means \pm SEM and frequencies (expressed as percentage). Statistical analyses were performed using S-PLUS 6.2 software. Occurrences of VT were compared with Cox's model and were analyzed as failure time data (rats without event were considered as censored). The assumption of proportional hazards between groups was confirmed, and the

group was the unique covariate selected in the Cox's model. Overall mortality between groups was compared using a Fisher's exact test. ECG parameters (P, RR, PR, QRS and QT and VERP values) were assessed by a linear mixed model with random slope and intercept in the control, vehicle, and myoblast groups. The fixed effects were the group and the time. Interaction between group and time was tested but not included in the model (not significant). Real time PCR data were assessed using the Kruskal-Wallis's test. Estimations were performed using the maximum likelihood. A value of $p < 0.05$ was considered significant.

RESULTS

Coronary ligation resulted in transmural myocardial infarction, as demonstrated by ECG recording immediately after ligation (ST segment elevation) and 2-5 days later (Q wave), troponin I dosage, and histological evaluation. Histomorphometric analysis of rat myocardium on 12 initial rats showed a reproducible scar of $25\pm 3\%$ of the left ventricle, 7 weeks after coronary ligation (Figure 1A). For electrophysiological studies, 81 animals underwent coronary ligation, and 25/81 (31%) died during surgery or before complete recovery. The 56 surviving animals were randomly divided into 3 groups: 1) a control group receiving no further treatment, 2) a vehicle group injected into the infarcted myocardium with culture medium at day 7 post-ligation, 3) a myoblast group injected with autologous myoblasts at day 7 post-ligation. None of the 56 surviving animals were lost between randomization and cell transplantation.

In Vivo Autologous Myoblast Implantation into Infarcted Myocardium

Autologous myoblasts were obtained by a 7-day culture of cells isolated from skeletal muscle biopsy. A total number of $14\pm 0.6 \times 10^6$ cells were injected, of which an average of $25\pm 3\%$ were identified as myoblasts by flow cytometry analyses and immunochemistry (Figure 1B). This range was similar to previous studies [5,13]. Cells were *in vitro* labeled with a fluorescent marker (CFSE), and immediately injected into the infarcted myocardium. Histological evaluation of the myocardium 24 hours after cell injection showed that most of the grafted cells were localized in the left ventricular wall around injection sites (Figures 1C, 1D). A few isolated grafted cells were also present throughout the left ventricle.

Electrophysiological Studies

Ambulatory ECG recordings in free-moving rats

Spontaneous arrhythmias related to myoblast transplantation were evaluated in a total of 1330 hr of telemetric recordings performed in the vehicle group (n=5, 597 hr) and in the myoblast group (n=5, 733 hr). During the first 48 hours following coronary ligation (5 days before cell transplantation) ventricular premature beats, couplets, triplets, and non sustained VT were observed in each rat (not shown). Only one animal demonstrated recurrent episodes of sustained and polymorphic VT (Figure 2A). This rat was randomly assigned to the vehicle group, and showed no further episode during the following recording periods.

Importantly, myoblast or vehicle injection at day 7 post-coronary ligation did not modify ECG morphology (Figure 2B). In both vehicle and myoblast groups, ventricular premature beats, couplets, and triplets were observed after myoblast or vehicle injection from day 7 to day 35 (Figure 2C). Nevertheless, these anomalies were rare and occurred with the same frequency in the vehicle and myoblast groups (i.e., triplets: 0.01 ± 0.01 per hour and in both groups, except in myoblast group at day 7 to day 14 (0.45 ± 0.42 per hour)). Finally, no episode of spontaneous sustained VT was observed in any recording performed from day 7 to day 35 after myoblast or vehicle injection.

Programmed Electrical Stimulation in sedated rats

Ventricular electrical instability related to cell transplantation was evaluated using PES procedure.

We first evaluated the feasibility and safety of repeated PES in sedated non-infarcted normal rats (n=4). Neither standard ECG measurements, nor VERP at 100-ms pacing cycle length were altered by the repeated PES procedures (day 14, 21, 28 and 35 post-electrode implantation; Table 1A). Importantly, PES did not trigger arrhythmias in any of these animals

(Figures 3A, 3B). Six weeks after electrode implantation, post-mortem macroscopic examination showed no evidence of tissue damage (data not shown).

In view of these results, the PES procedure was then conducted in infarcted rats, at day 14, 21, 28 and 35 post-coronary ligation. No differences between groups were observed in ECG parameters prior to the first PES procedure (day 14). As in non-infarcted rats, neither standard ECG measurements, nor VERP at 100-ms pacing cycle length, were significantly altered by the repeated PES procedures (linear mixed model; Table 1B).

In contrast to non-infarcted rats, PES induced sustained VT in all groups of rats with myocardial infarction. ECG parameters of rats from the myoblast group that underwent sustained VT during PES did not differ from those of myoblast group rats that did not show sustained VT (not illustrated). This data suggests that ECG parameters (and more particularly QRS duration) are not a predictive factor for arrhythmia induction.

Triggered sustained VT were monomorphic and the morphology was similar in a same animal from day 14 to day 35 (Figure 4A). VT cycle lengths ranged from 65 to 90 ms. The percentage of animals experiencing at least one episode of sustained VT during one of the 4 PES procedure, was similar between the control and vehicle groups (29% and 21%, respectively; $p=0.50$, Cox's model, Figure 5A). In striking contrast, 65% of the animals from the myoblast group showed at least one episode of sustained VT during PES ($p<0.05$ versus control group and $p<0.005$ versus vehicle group, Cox's model). In the control and vehicle groups, the number of newly inducible rats remained stable over time (Figure 5B). In contrast, the first episode of sustained VT occurred at day 14 or 21 in 70% of the rats from the myoblast group, suggesting that increased susceptibility of sustained VT in the myoblast group was transient.

As a further control group, we performed autologous bone marrow mononuclear cell transplantation into the infarcted myocardium of 9 additional rats (BM-MNC group). Animals included in the BM-MNC group could not be randomized, since muscle biopsy was not performed in this group to avoid further increase in mortality. However, the number of injections, the injection locations, the injected volume, and the number of injected BM-MNC were similar to that of myoblast transplantation, and PES were performed by an operator blinded to treatment. Histological evaluation of myocardium performed 24 hour after BM-MNC transplantation confirmed that most of the grafted cells were localized in the left ventricular wall within the infarct size, around the injection site (Figure 1E, 1F) as observed in the myoblast group. In marked contrast, in this BM-MNC group, only 3/9 rats experienced at least one episode of sustained VT during one of the 4 PES, a number similar to that of the control and vehicle groups (Figure 5). This finding suggests that cell injection *per se* does not increase electrical instability, and that myoblasts exhibit a specific arrhythmogenic risk.

Monomorphic sustained VT terminated spontaneously in 92% of the cases or degenerated into ventricular fibrillation (VF; Figure 4B). Such VF were observed in 4/20 rats of the myoblast group, resulting in one death. In 2 other animals VF was stopped by a blow on the chest, and in the remaining VF resumed spontaneously. VF was observed in only 1/17 rats from the control group, 1/9 rats from the BM-MNC group, and 0/19 rats from the vehicle group.

Overall, the mortality rate was similar in all groups (*i.e.* 41%, 37%, 40% and 30% for control, vehicle, myoblast and BM-MNC groups, respectively; $p=1$, Fisher's exact test). With the exception of the VF-induced death in the myoblast group, all other deaths were observed during recovery from anesthesia and coincided with marked bradycardia in animals in which an ECG was recorded. Post-mortem examination showed no sign of myocardial rupture or pulmonary edema.

Hypertrophic Response and Myotube Differentiation

Rats were sacrificed 6-7 weeks after coronary ligation (i.e., 5-6 weeks after cell injection). The hypertrophic response to myocardial infarction was measured by relative quantification of ANF, β -MHC and α -actin mRNA transcripts using TaqMan real-time PCR [14]. A significant increase in ventricular ANF, β -MHC and α -actin mRNA expression, confirmed ventricular hypertrophy due to myocardial infarction in coronary ligated rats compared to sham rats ($p < 0.02$). However no significant difference of mRNA expression were observed among groups ($p = 0.17$; 0.21 and 0.36 for ANF, β -MHC and α -actin, respectively; Kruskal-Wallis's test; Figure 6A). This result suggests that difference in VT induction between groups was not related to ventricular hypertrophy.

Histological analyses and immunohistochemistry against fast skeletal myosin heavy chain, a specific marker for myotubes, in rats injected with autologous myoblasts at 5 weeks after myoblast injection demonstrated the presence of multinucleated myotubes within the infarction scar (Figures 6B, 6C, 6D). This showed that transplanted cells were still present and had differentiated into skeletal myotubes. In order to evaluate gap junction formation between host and transplanted cells, double immunolabeling of fast skeletal myosin heavy chain and connexin43 were performed in myoblast transplanted myocardium 5 weeks after myoblast injection. Connexin43 was detected between neighboring host cardiomyocytes at cardiomyocyte junctional borders. In contrast no connexin43 was detected between transplanted myotubes and host cardiomyocytes (Figure 6E), suggesting the lack of gap junctions between host cardiomyocytes and transplanted cells.

DISCUSSION

Our results demonstrate that (1) medium or cell injection does not facilitate *per se* the occurrence of tachyarrhythmias, (2) myoblast transplantation into injured myocardium induces electrical instability with triggering of sustained ventricular tachycardia, (3) arrhythmias due to myoblast transplantation are transient despite the persistence of the transplanted myoblasts, (4) electrophysiological evaluation with PES may be necessary to identify potential arrhythmogenic risk of cardiac cell therapy in rodent models.

Small scale clinical trials demonstrated the feasibility and the efficacy of autologous myoblast transplantation to improve ventricular function after myocardial infarction, but also revealed a high incidence of cardiac ventricular arrhythmias, suggesting that myoblast transplantation by itself could be arrhythmogenic [3,15]. Our observations are in good agreement with these clinical studies, showing electrical instability within weeks following myoblast transplantation.

Using surface ECG recording in a mouse model, Koh *et al.* failed to detect arrhythmias after skeletal myoblast transplantation into a non-injured myocardium [16]. However, this result should be considered with caution because of the depth of anesthesia and the cardiac injury lack that could lower the risk of arrhythmias. Moreover, because arrhythmias are highly unpredictable, analyses of short periods of ECG recordings may not be sufficient to detect them. On the other hand, our analyses of numerous hours of telemetry recording also failed to detect sustained VT in myoblast and vehicle groups. In large animal models such as cardiomyopathic dogs [17] and coronary ligated rabbits [18], ECG recordings showed erratic and aggressive ventricular ectopy, and lethal bradycardia after autologous myoblast transplantation. However, in these studies as in the initial clinical trials, in the absence of control group it was difficult to connect arrhythmias directly to myoblast transplantation,

since heart failure or tissue injury (due to disease-inducing surgical procedure and/or to needle puncture) could be responsible for such arrhythmogenesis. This discrepancy in the occurrence of spontaneous arrhythmias between rodents and larger animal models might be due to the different cardiac electrophysiological properties of these models, in particular the fast cardiac rhythm in rodents. Nevertheless, this demonstrates that Holter monitoring is not reliable to identify electrical instability in rodent models after cardiac cell therapy, and this suggests that this may not be reliable to assess the arrhythmogenic risk of cell, gene or protein injection into the myocardium.

In order to further evaluate electrical instability related to myoblast transplantation, we used *in vivo* electrophysiological testing with PES. Non-invasive PES procedure allows repeated studies and easy follow-up of the natural course arrhythmogenesis. With this approach, we clearly showed that intracardiac myoblast injection increases electrical instability. Moreover, despite an important mortality rate due to the repeated PES procedure, such electrical instability seems to be transient. Importantly, analyses of ECG parameters, including QRS duration, did not show any ECG predictive factor for arrhythmogenic risk. These results further demonstrate the need for relevant preclinical models to assess the risk of life-threatening ventricular arrhythmias after cell, gene or protein injection into the myocardium.

Vehicle-injected animals did not show higher incidence of cardiac arrhythmias than control (non injected) animals, suggesting that ventricular injection *per se* is not arrhythmogenic, as reported with other pro-inflammatory therapies such as direct laser transmyocardial revascularization [2]. Moreover, RT-PCR study performed in a small group of animals showed that control animals did not have higher ventricular hypertrophy than myoblast injected animals, suggesting that arrhythmias do not reflect the natural history of myocardial infarction. Local cell injection induces highly uneven distribution of cells, which may also

increase electrophysiological heterogeneity and favor reentrant mechanisms [2]. Importantly, distribution of cell into the myocardium after intramyocardial injection was similar after myoblast or bone marrow mononuclear cell injection whereas, in contrast to myoblast intramyocardial injection, intramyocardial injection of autologous bone marrow mononuclear cells did not increase the incidence of ventricular arrhythmias. This absence of ventricular tachycardia was previously observed in clinical trials using bone marrow mononuclear cells [19]. All these observations suggest that myoblasts exhibit a specific arrhythmogenic mechanism. Importantly, VT were induced during PES despite probable poor cell survival [20].

In the present study, only 25% of injected cells after skeletal muscle primary culture were myoblasts at the time of transplantation, and ventricular tachycardia might result from the presence of non-myoblast cells [21]. However, in clinical trials where tachyarrhythmias were first observed, the percentage of injected myoblasts was significantly higher than in our experimental study (55% to 80%) [4,15,22]. This suggests that higher percentage of myoblast in injected cells does not decrease arrhythmogenic risk.

Transplanted myoblasts did not form gap junctions *in vivo* with the host myocardium, even when they were in direct physical contact with host cardiomyocytes, a result already shown in previous studies [23,24]. Transplanted myoblasts differentiate into peculiar hyperexcitable myotubes with a contractile activity fully independent of neighboring cardiomyocytes [5]. Upon rapid external stimulation, these myotubes develop tetanus, indicating that they retain functional properties of skeletal myotubes [25]. The direct cause of cardiac hyperexcitability following myoblast transplantation remains unknown, but the lack of connexin expression and the failure of skeletal muscle cells to couple to neighboring cardiac myocytes may play an important role [26]. Recent *in vitro* study demonstrated that coculture of skeletal myoblasts

and cardiomyocytes resulted in reentrant arrhythmias and that such arrhythmias could be limited by overexpression of connexin43 [27], suggesting the lack of gap junction as a possible mechanism for ventricular arrhythmias.

Notwithstanding the aforescribed data, the present report has limitations. The present study does not clarify why autologous myoblast transplantation increases the inducibility of ventricular arrhythmias without an increased prevalence of spontaneous arrhythmias. However, small rodents are actually well known - and this is confirmed by our telemetric recordings - for their low incidence of benign arrhythmias such as extrasystoles [28]. This can be partly explained by different electrophysiological properties when compared to human heart, and by the high sinus rate which overdrives potential arrhythmogenic foci. Another limitation of the study was the fact the group of rats treated with autologous bone marrow mononuclear cell transplantation was not randomized at the beginning of the study, but was added during the course of the study. However, all analyses of electrophysiologic data were performed by operators blinded to treatment group and during the same periods of time, suggesting that this did not compromise the study.

Finally, our study enlightens ventricular arrhythmias as a potential serious limitation of myoblast and stem cell therapies. Future pre-clinical and clinical studies will determine the most effective and safer cell types for myocardial repair, and the clinical significance of cell therapy-induced arrhythmias. Because ventricular arrhythmias are life-threatening diseases, it will be important to evaluate other cell sources for cardiac cell therapy in *in vivo* experimental electrophysiological models before clinical use. It is also crucial to further evaluate the potential mechanisms of arrhythmias and the strategies to control and eliminate these arrhythmias.

Acknowledgments: We thank Jean-Thomas Vilquin for his help with myoblast cell culture and Jean-Christophe Chevallier for his help with electrophysiological studies. This work was supported, in part, by INSERM Avenir grant, by the Association Française contre les Myopathies, by the GIS-maladies rares, and by the Fondation pour la Recherche Médicale (INE20030307053).

FIGURE LEGENDS

Figure 1. Myocardial infarction and cell transplantation. A: Sirius red staining of ventricular myocardium, in a rat 5 weeks after coronary ligation. Arrows show a necrotic transmural scar (red color). B: Detection of myoblasts in *in vitro* muscle-derived expanded cells, using immunolabeling of desmin, a specific marker for myoblasts (pink). Nuclei were stained with propidium iodide (blue). C to F: Detection of transplanted cells in a frozen ventricular section, 24 hours after transplantation. Cells were labeled prior to transplantation with a fluorescent cytoplasmic marker, CFSE (green fluorescence, arrowheads). Cell nuclei were labeled with propidium iodide (red fluorescence). C: Myoblast transplantation, the area in the rectangle is also shown at higher magnification (D). E: Bone Marrow Mononuclear Cell transplantation, the area in the rectangle is also shown at higher magnification (F). A, C and E, bars=1 mm. B, D and F, bars=100 μ m.

Figure 2. Telemetric recordings. A: Spontaneous sustained ventricular tachycardia at day 0, 4 hours after coronary ligation and 7 days before vehicle or myoblast injection. B: Representative telemetric recordings obtained at day 7 in one animal, immediately before (A) and after (B) myoblast injection. Ventricular extrasystoles (blue arrows) without VT were observed in both cases. C: Spontaneous triplet and quadruplet observed during telemetric recording after myoblast transplantation at day 14 post-coronary ligation.

Figure 3. Pilot study of Programmed Electrical Stimulation (PES) in normal rats, without coronary ligation. Representative lead II (LII) surface ECGs showing the effects of a train of 9 paced beats at a cycle length of 100 ms (S1, red arrows), followed by 3 extrastimuli (S2, S3, S4, blue arrows), at day 14 (A) and 35 (B) post-electrode implantation. Repeated PES procedures did not trigger any sustained arrhythmia.

Figure 4. Ventricular arrhythmias during Programmed Electrical Stimulation (PES) procedure. A: Representative surface 3-lead ECG in a rat from the myoblast group, showing the induction of self-terminating ventricular tachycardia with a train of 9 paced beats at a cycle length of 100 ms, followed by 2 extrastimuli. B: Surface lead I ECG in a rat from the myoblast group, showing the induction of sustained ventricular tachycardia degenerating into ventricular fibrillation (red arrow). Pacing protocol consisted in a train of 9 stimuli at a cycle length of 100 ms, followed by 3 extrastimuli. Fibrillation was stopped by a blow on the chest (blue arrow).

Figure 5. Myoblast-induced ventricular hyperexcitability. Rats underwent Programmed Electrical Stimulation (PES) procedure. A: Total number of rats with at least 1 episode of sustained ventricular tachycardia (VT) at day 14 to 35 post-coronary ligation ($p < 0.05$ myoblast *versus* control group, $p < 0.005$ myoblast *versus* vehicle group). B: First episode of sustained VT in rats during PES, performed at day 14, 21, 28 and 35 post-coronary ligation.

Figure 6. Hypertrophic response and myotube differentiation. A: Hypertrophic response to myocardial infarction, 6 weeks after coronary ligation, measured by Atrial Natriuretic Factor (ANF), β -Myosin Heavy Chain (β -MHC), and α -actin gene expression in the ventricular myocardium of rats using TaqMan real time RT-PCR. ANF, β -MHC and α -actin gene expression were corrected by HPRT gene expression levels. Gene expression in non-operated rats (Sham) was used as baseline level. * $p < 0.02$; NS: not significant. B: Detection of myotubes 5 weeks after myoblast transplantation in a ventricular section, by immunolabeling against fast skeletal MHC (green fluorescence). Cell nuclei were labeled with propidium iodide (red fluorescence). C: Higher magnification of the previous panel. D: Higher magnification showing one striated (arrows) myotube. E: Connexin43 (blue fluorescence) and myotube (green fluorescence) immunolabelings 5 weeks after myoblast

transplantation. Arrows indicate a multinucleated myotube, and arrowheads connexin43. Cell nuclei were labeled with TO-PRO3 (red fluorescence). B and C, Bars=100 μm ; D, Bar=5 μm ; E, Bar=10 μm .

TABLES

Table 1. ECG measurements: ECG measurements were performed under sinus rhythm. Ventricular effective refractory period (VERP) was measured at a basic pacing cycle length (BCL) of 100 ms, (A) at day 14, 21, 28 and 35 after electrode implantation in non-infarcted rats (pilot group), and (B) at day 14, 21, 28 and 35 after coronary ligation in control, vehicle and myoblast groups.

Table 1A

Time	n	ECG parameters (ms)					VERP	
		RR	P	PR	QRS	QT	(BCL=100 ms)	
Day 14	4	153±8	20±1	50±2	18±1	68±5	67±5	
Day 21	4	161±6	19±0	48±2	20±0	76±2	68±4	
Day 28	4	154±9	20±0	53±3	20±1	74±2	60±2	
Day 35	4	147±1	20±1	47±4	20±1	69±2	51±9	

Table 1B

Time	Groups	n	ECG parameters (ms)					VERP	
			RR	P	PR	QRS	QT	(BCL=100 ms)	
Day 14	Control	17	151±4	19±1	51±1	19±1	74±2	70±3	
	Vehicle	19	150±3	19±0	49±1	19±1	70±3	64±2	
	Myoblast	20	154±4	20±1	51±2	20±0	72±2	67±3	
Day 21	Control	17	152±4	19±1	50±1	20±0	74±3	67±3	
	Vehicle	19	159±4	19±0	50±1	19±1	73±3	66±3	
	Myoblast	18	157±4	19±0	50±1	19±1	76±2	62±2	
Day 28	Control	13	164±13	19±1	52±2	20±0	75±3	73±5	
	Vehicle	14	151±5	19±0	50±1	20±1	71±2	64±2	
	Myoblast	13	152±4	20±1	51±1	19±1	71±2	61±2	
Day 35	Control	10	153±8	20±1	51±2	20±1	64±4	64±4	
	Vehicle	12	155±6	19±0	51±1	19±1	72±2	66±2	
	Myoblast	12	143±4	19±1	50±1	20±1	72±1	64±2	
p value			0.83	0.49	0.60	0.74	0.86	0.18	

Abbreviations are: P, P wave duration; RR, PR, QRS and QT are RR, PR, QRS and QT intervals respectively. All measurements were performed under general anesthesia on lead I. Results are expressed as mean±SEM.

BIBLIOGRAPHY

1. Menasche P, Hagege AA, Scorsin M, Pouzet B, Desnos M, Duboc D, *et al.* Myoblast transplantation for heart failure. *Lancet* 2001;357:279-80.
2. Makkar RR, Lill M, Chen PS. Stem cell therapy for myocardial repair: is it arrhythmogenic? *J Am Coll Cardiol* 2003;42:2070-2.
3. Menasche P. Skeletal muscle satellite cell transplantation. *Cardiovasc Res* 2003;58:351-7.
4. Smits PC, van Geuns RJ, Poldermans D, Bountiukos M, Onderwater EE, Lee CH, *et al.* Catheter-based intramyocardial injection of autologous skeletal myoblasts as a primary treatment of ischemic heart failure: clinical experience with six-month follow-up. *J Am Coll Cardiol* 2003;42:2063-9.
5. Leobon B, Garcin I, Menasche P, Vilquin JT, Audinat E, Charpak S. Myoblasts transplanted into rat infarcted myocardium are functionally isolated from their host. *Proc Natl Acad Sci U S A* 2003;100:7808-7811.
6. Bigger JT, Jr., Whang W, Rottman JN, Kleiger RE, Gottlieb CD, Namerow PB, *et al.* Mechanisms of death in the CABG Patch trial: a randomized trial of implantable cardiac defibrillator prophylaxis in patients at high risk of death after coronary artery bypass graft surgery. *Circulation* 1999;99:1416-21.
7. Zhang M, Methot D, Poppa V, Fujio Y, Walsh K, Murry CE. Cardiomyocyte grafting for cardiac repair: graft cell death and anti-death strategies. *J Mol Cell Cardiol* 2001;33:907-21.
8. Bottino R, Lemarchand P, Trucco M, Giannoukakis N. Gene- and cell-based therapeutics for type I diabetes mellitus. *Gene Ther* 2003;10:875-89.

9. Pouzet B, Vilquin JT, Hagege AA, Scorsin M, Messas E, Fiszman M, *et al.*
Intramyocardial transplantation of autologous myoblasts: can tissue processing be optimized?
Circulation 2000;102:210-5.
10. Bronner-Fraser M. Alterations in neural crest migration by a monoclonal antibody that affects cell adhesion. *J Cell Biol* 1985;101:610-7.
11. Walker MJ, Curtis MJ, Hearse DJ, Campbell RW, Janse MJ, Yellon DM, *et al.* The Lambeth Conventions: guidelines for the study of arrhythmias in ischaemia infarction, and reperfusion. *Cardiovasc Res* 1988;22:447-55.
12. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25:402-8.
13. Al Attar N, Carrion C, Ghostine S, Garcin I, Vilquin JT, Hagege AA, *et al.* Long-term (1 year) functional and histological results of autologous skeletal muscle cells transplantation in rat. *Cardiovasc Res* 2003;58:142-8.
14. Fraccarollo D, Bauersachs J, Kellner M, Galuppo P, Ertl G. Cardioprotection by long-term ET(A) receptor blockade and ACE inhibition in rats with congestive heart failure: mono-versus combination therapy. *Cardiovasc Res* 2002;54:85-94.
15. Menasche P, Hagege AA, Vilquin JT, Desnos M, Abergel E, Pouzet B, *et al.*
Autologous skeletal myoblast transplantation for severe postinfarction left ventricular dysfunction. *J Am Coll Cardiol* 2003;41:1078-83.
16. Koh GY, Klug MG, Soonpaa MH, Field LJ. Differentiation and long-term survival of C2C12 myoblast grafts in heart. *J Clin Invest* 1993;92:1548-54.
17. Borenstein N, Chetboul V, Rajnoch C, Bruneval P, Carpentier A. Successful cellular cardiomyoplasty in canine idiopathic dilated cardiomyopathy. *Ann Thorac Surg* 2002;74:298-9.

18. Soliman AM, Taylor DA, Thompson RB, Morimoto Y, McMichael M, Crater S, *et al.* Assessment of electrical instability after autologous myoblast transplantation in a rabbit infarct model. *Circulation* 2003;108(Suppl IV):547-8 (abstract).
19. Haider H, Ashraf M. Bone marrow stem cell transplantation for cardiac repair. *Am J Physiol Heart Circ Physiol* 2005;288:H2557-67.
20. Azarnoush K, Maurel A, Sebbah L, Carrion C, Bissery A, Mandet C, *et al.* Enhancement of the functional benefits of skeletal myoblast transplantation by means of coadministration of hypoxia-inducible factor 1alpha. *J Thorac Cardiovasc Surg* 2005;130:173-9.
21. Lee MS, Makkar RR. Stem-cell transplantation in myocardial infarction: a status report. *Ann Intern Med* 2004;140:729-37.
22. Pagani FD, DerSimonian H, Zawadzka A, Wetzel K, Edge AS, Jacoby DB, *et al.* Autologous skeletal myoblasts transplanted to ischemia-damaged myocardium in humans. Histological analysis of cell survival and differentiation. *J Am Coll Cardiol* 2003;41:879-88.
23. Reinecke H, MacDonald GH, Hauschka SD, Murry CE. Electromechanical coupling between skeletal and cardiac muscle. Implications for infarct repair. *J Cell Biol* 2000;149:731-40.
24. Horackova M, Arora R, Chen R, Armour JA, Cattini PA, Livingston R, *et al.* Cell transplantation for treatment of acute myocardial infarction: unique capacity for repair by skeletal muscle satellite cells. *Am J Physiol Heart Circ Physiol* 2004;287:H1599-608.
25. Rubart M, Soonpaa MH, Nakajima H, Field LJ. Spontaneous and evoked intracellular calcium transients in donor-derived myocytes following intracardiac myoblast transplantation. *J Clin Invest* 2004;114:775-83.
26. van den Bos EJ, Davis BH, Taylor DA. Transplantation of skeletal myoblasts for cardiac repair. *J Heart Lung Transplant* 2004;23:1217-27.

27. Abraham MR, Henrikson CA, Tung L, Chang MG, Aon M, Xue T, *et al.*
Antiarrhythmic Engineering of Skeletal Myoblasts for Cardiac Transplantation. *Circ Res*
2005 (In Press).
28. Nerbonne JM. Studying cardiac arrhythmias in the mouse--a reasonable model for
probing mechanisms? *Trends Cardiovasc Med* 2004;14:83-93.

Figure 1

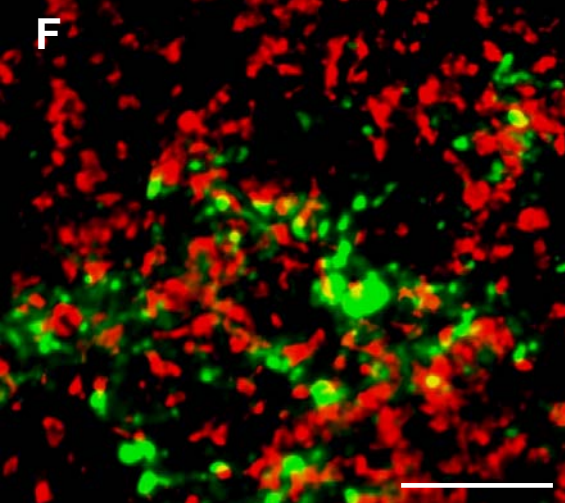
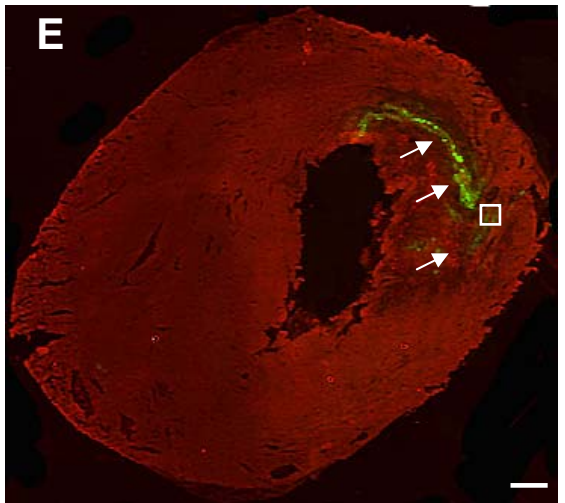
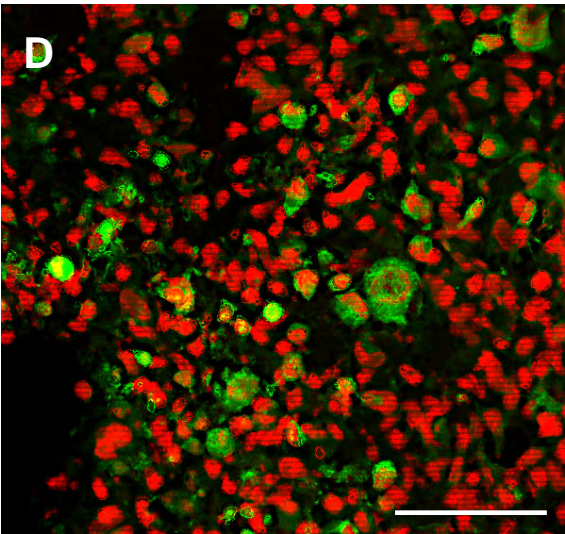
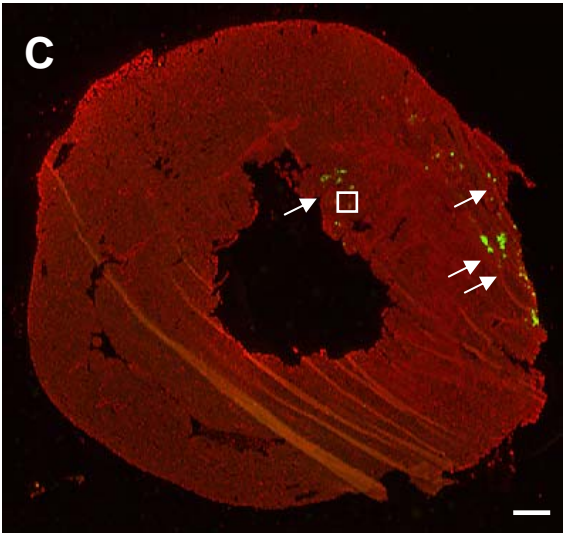
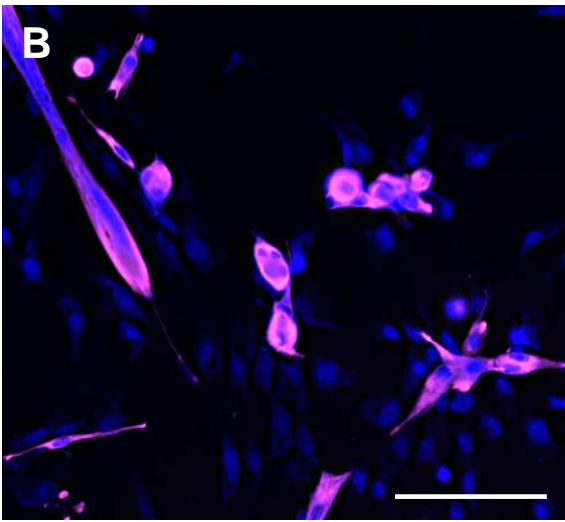
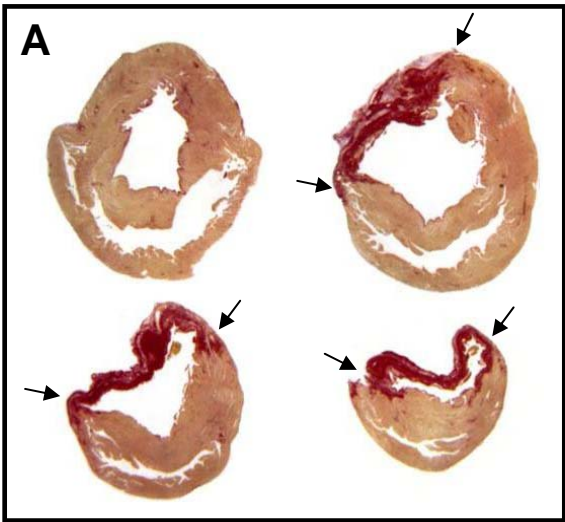


Figure 2

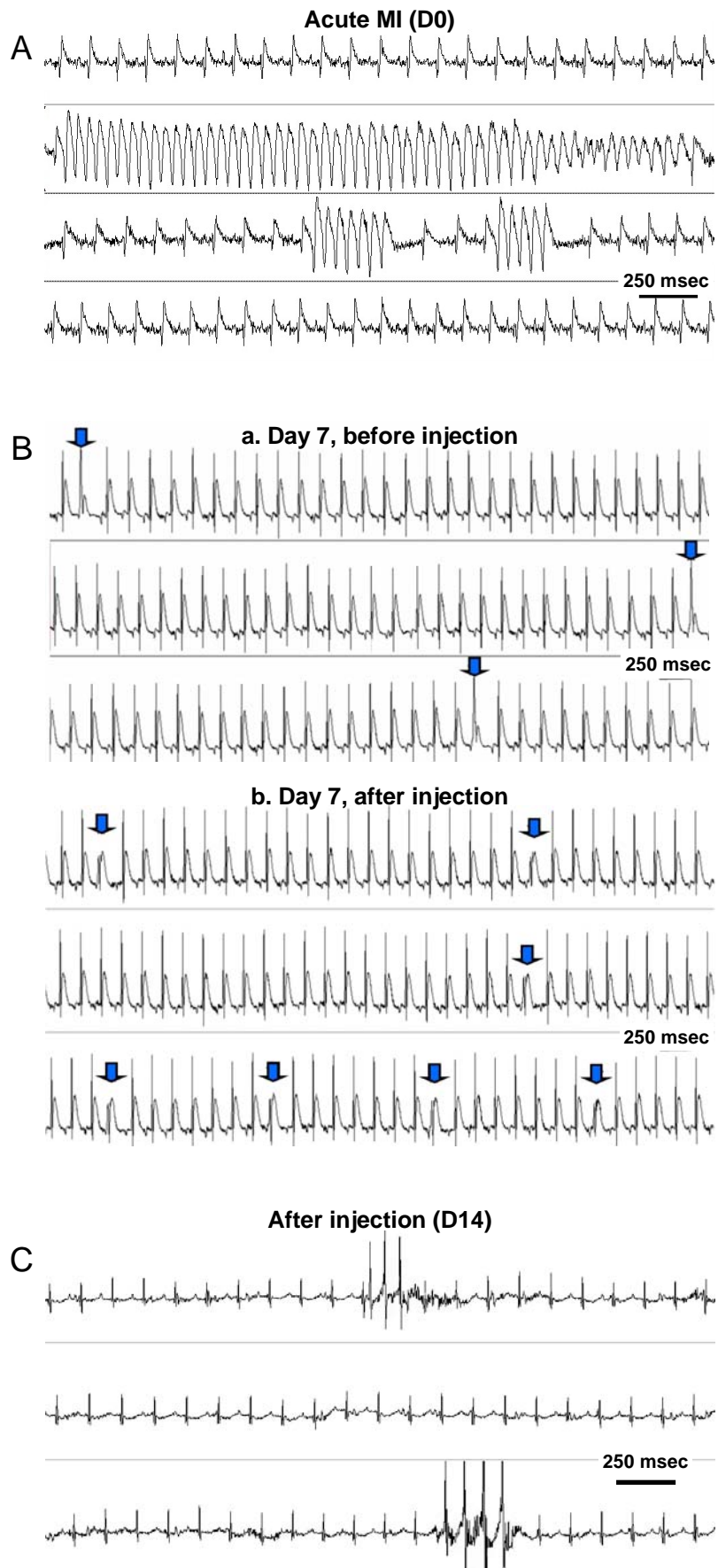


Figure 3

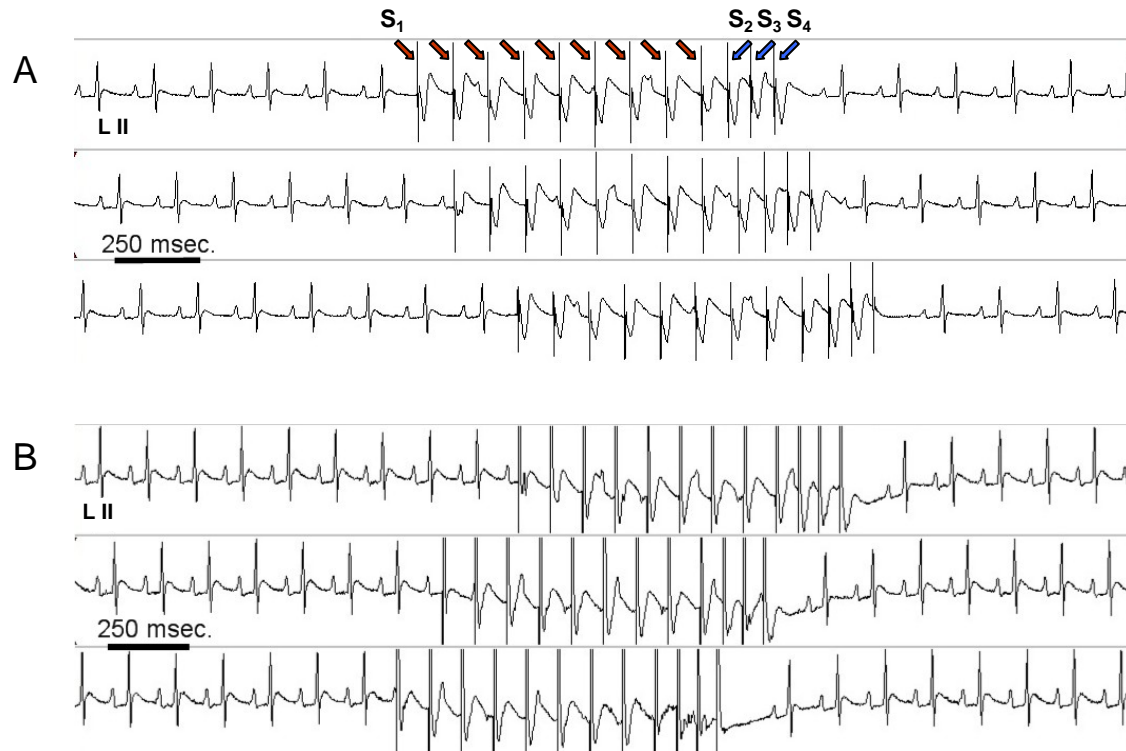


Figure 4

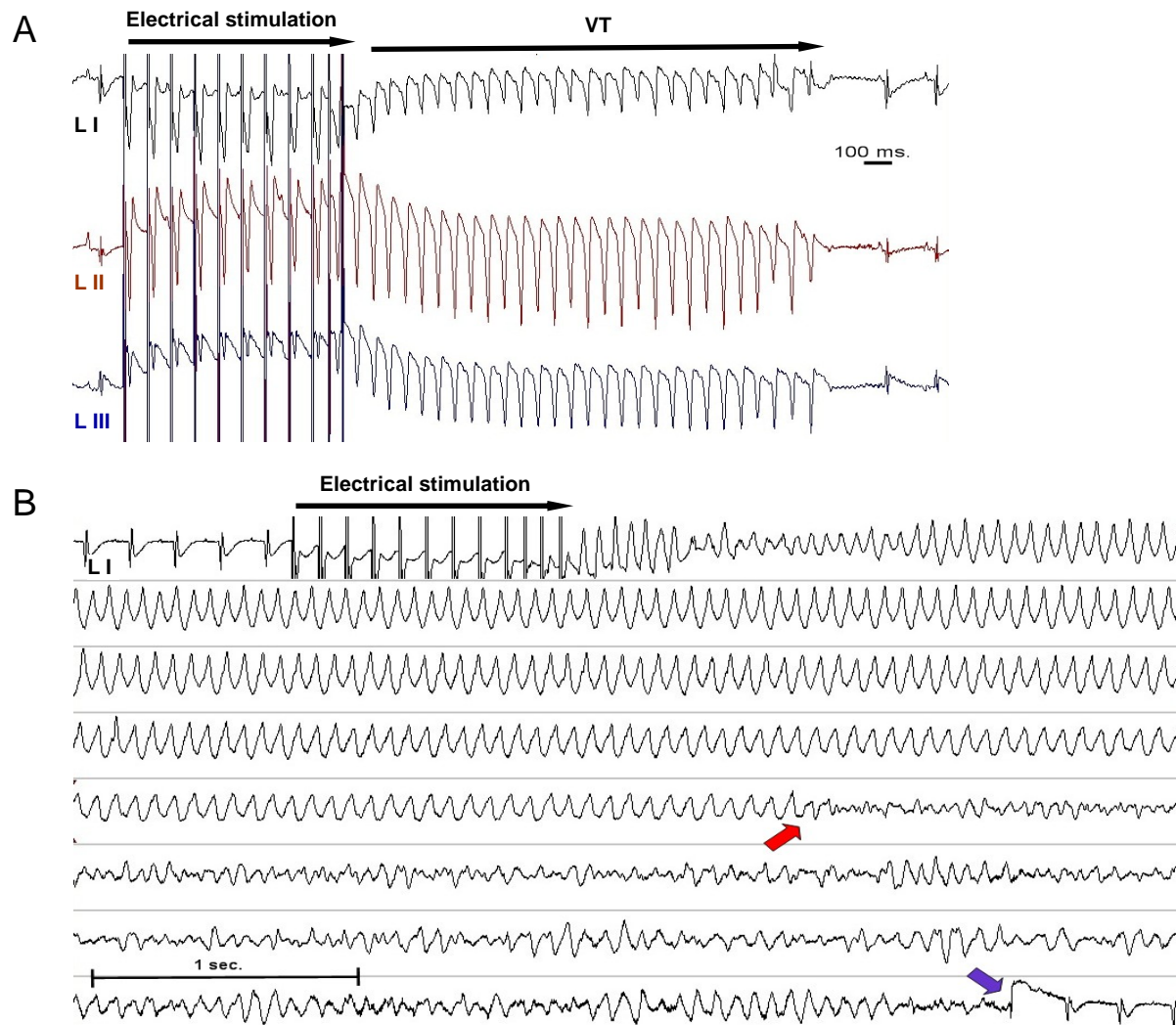


Figure 5

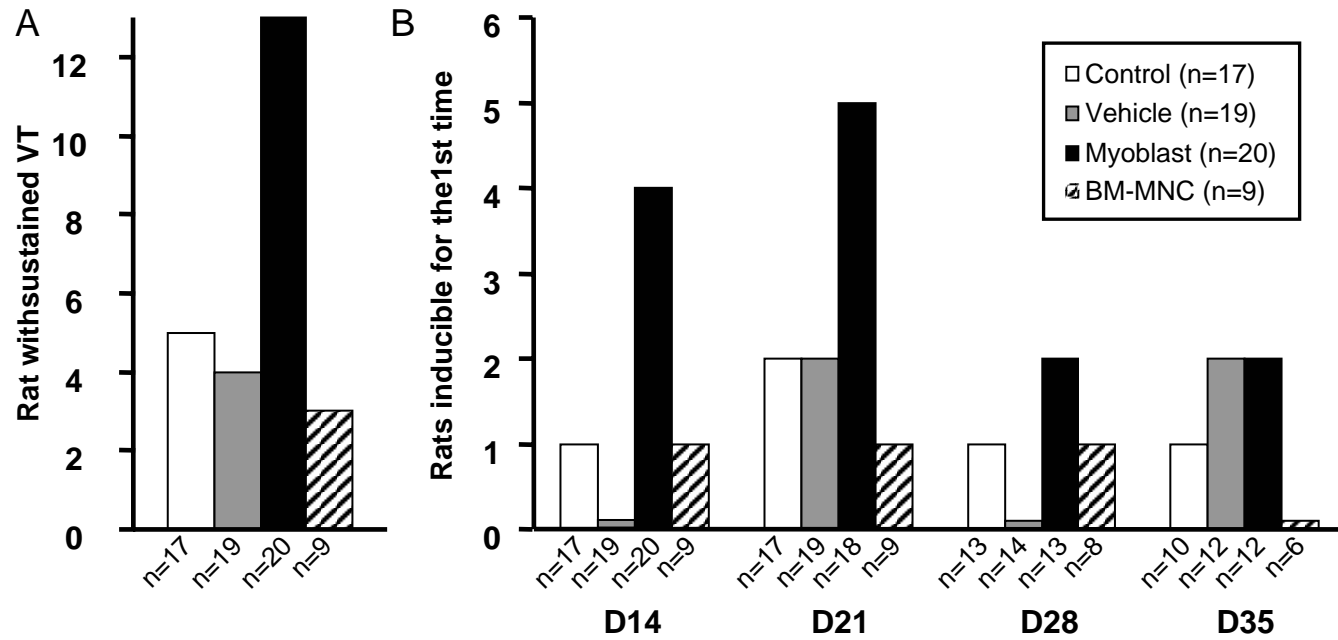


Figure 6

