Intramyocardial Transplantation of Autologous Myoblasts: Can Tissue Processing Be Optimized?

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Background—Autologous skeletal myoblast (SM) transplantation improves function of infarcted myocardium, but pretransplantation cultures remain a complex process. This study assessed whether it could be optimized by muscle preconditioning with the local anesthetic bupivacaine or even bypassed with the use of the so-called mince technique.

Methods and Results—Muscle preconditioning consisted of intramuscular injections of the tibialis anterior of rats, 2 days before harvest. After 7 days of culture, the number of available myoblasts was significantly increased compared with nonconditioned controls (1 683 147 versus 85 300, P=0.0013). The mince technique was then assessed. A myocardial infarction was created in 66 rats by coronary artery ligation. One week later, rats were reoperated on and intramyocardially injected with culture medium alone (controls, n=23), autologous cultured SM (3.5×10⁶, n=21), or autologous muscle minced into a fine slurry, which was immediately transplanted (n=22). All muscles had been preconditioned. Left ventricular function was assessed by 2D echocardiography. Whereas end-diastolic volumes expanded over time in all groups, left ventricular ejection fraction (%, mean±SEM) was increased only in the cultured SM–transplanted group at 1 (P=0.0006) and 2 months (P=0.0008) versus baseline (37.5±1.92 and 40.9±2.17 versus 30.34±1.74), with a significant additional benefit between 1 and 2 months (P=0.0069).

Conclusions—Cell culture remains mandatory for SM transplantation to be successful but, in a clinical perspective, this process can be made more expeditious by preharvest muscle conditioning with bupivacaine, which greatly enhances the baseline cell yield. (Circulation. 2000;102[suppl III]:III-210-III-215.)

Key Words: heart failure — transplantation — infarction — cells — muscles

Intramyocardial transplantation of contractile cells is increasingly recognized as an effective means of repairing ischemically damaged hearts. Initial research has focused on fetal cardiomyocytes, but clinically relevant issues related to the availability and immunogenicity of these cells have then led us to consider skeletal myoblasts (also known as satellite cells) as alternate candidates for intramyocardial engraftment.

Using a rabbit model of cryonecrosis, Taylor and coworkers1 have documented a significant improvement in function after autologous skeletal myoblast transplantation. The use of these cells, which offer the tremendous advantage inherent in autografts, is now further supported by recent data from our laboratory2 showing that the functional capabilities of skeletal myoblasts are equivalent to those of fetal cardiomyocytes.

Regardless of the nature of the cells to be grafted, one of the key factors of a successful functional outcome is the adequacy of their processing. This processing basically encompasses isolation of the contractile (cardiac or skeletal) elements followed by their expansion through multiple passages. In the perspective of clinical applications, however, cultures raise several issues including duration of the period required for yielding a substantial number of cells, repeated manipulations with an attendant risk of infectious contamination, logistical constraints related to the back-and-forth transportation of the samples between the harvest/transplant center and the cell culture facilities, and ultimately, cost.

The present study was therefore designed to assess if, and how, this process could be optimized. The first objective was to investigate the effect of muscle preconditioning, with the use of an intramuscular injection of the local anesthetic bupivacaine, which induces a degeneration-regeneration cycle and thus promotes myoblast proliferation.3 The second objective was to assess whether cultures could be bypassed, with the use of the so-called mince technique. This hypothesis was based on the fact that mincing has been successfully used for transplanting fetal tissue into the brain of patients with Parkinson’s disease.4

Methods

All experiments were performed in accordance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals. All procedures were approved by the ethical committee of the hospital concerned.


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**Myocardial Infarction Model**

Male Wistar rats, weighing 280 g, were anesthetized with ketamine (50 mg/kg IP) and xylazine (10 mg/kg IP) and tracheally ventilated at 55 cycles/min with 2.5 mL tidal volume (14-gauge intravenous catheter intubation, Harvard Rodent Ventilator, Harvard Apparatus Co, Inc). They underwent a left lateral thoracotomy in the fifth intercostal space, and after exposure of the heart, the myocardial infarction was created by ligation of the left anterior descending branch of the left coronary artery, between the left atrium and the right pulmonary outflow tract, with a 7/0 polypropylene snares (Ethicon, Inc).

**Functional Assessment**

One week after myocardial infarction, and 1 and 2 months after transplantation, left ventricular function was studied by 2D echocardiography.

Under general anesthesia with ketamine (50 mg/kg IP) and xylazine (10 mg/kg IP), the rat’s chest was shaved and a layer of acoustic coupling gel was applied to the thorax; 2D (and M-mode) measurements were performed with a commercially available 15-MHz (15SL8) linear-array transducer system (Sequoia, Acuson Corp), allowing a 160-Hz maximal frame rate. The animals were placed in the supine or lateral decubitus position on a warming table, and care was taken to avoid excessive pressure on the thorax, which can induce severe bradycardia. Para-sternal long-axis views were obtained, making sure that the mitral and aortic valves and the apex were well visualized, and were then recorded.

Measurements of maximal left ventricular (LV) long-axis lengths (L) and endocardial area tracings (a), with the leading edge method, were performed from digital images captured on cine loops. Left ventricular end-diastolic volume (LVEDV) and left ventricular end-systolic volume (LVESV) were calculated by the single-plane area-length method: $V = \frac{8 \times a^2}{3 \times \pi \times L}$, $L$ = LVESV/LVEDV. All measurements were determined by 2 investigators who were blinded to the treatment groups and were made from ≥3 beats.

**Skeletal Muscle Preconditioning**

Forty-eight hours before muscle harvest, 0.5 mL bupivacaine (bupivacaine chloride 0.5%, Marcaine, Astra) was injected into the tibialis anterior muscles of rat hind limbs. To assess the efficacy of these injections, a specific set of experiments was designed. Four rats were randomly injected with bupivacaine in the left or right tibialis anterior muscle. The contralateral muscle was left intact and served as a control. Both muscles were harvested 48 hours later, as described below. Tibialis anterior muscles of each rat were used for primary cell cultures during 1 week, and a cellular count was performed at that time (see techniques below).

**Cell Culture Methodology**

**Isolation and Initial Plating of Skeletal Myoblasts**

During the myocardial infarction procedure (immediately before thoracotomy), the right and left tibialis anterior muscles were dissected, taking care of the extensor digitorum longus just behind, and harvested to remove the tendon and the aponeurotic tissue from the muscle tissue. The muscles were roughly cut and maintained in nutrient mixture F12(Ham), (GIBCO BRL). Then, they were minced, weighed, and enzymatically dissociated with collagenase IA (2 mg/mL, Sigma Chemical Co) for 1 hour and trypsin-ENDTA (0.25%, GIBCO BRL) for 20 minutes.

The cells were collected by sedimentation (7 minutes at 1200 rpm), and the enzyme reaction was arrested by adding 10% FBS (HyClone). After passage through a 100-μm sieve (Cell Strainer Nylon, Becton Dickinson) and centrifugation, the supernatant was discarded and the cells were resuspended in the culture medium composed of F12(Ham) with 20% FBS (vol/vol), 1% (vol/vol) penicillin-streptomycin (10000 UI/mL–10000 μg/mL, GIBCO BRL), and 5 mg/mL basic fibroblast growth factor (Sigma Chemical Co).

Initial plating was realized in 75-cm² tissue culture flasks (Falcon, Becton Dickinson), and cells were grown in humid air with 5% CO₂.

**In Vitro Cell Multiplication**

One day later, the cells were washed and the growth medium was changed. Before confluence, the cells were split into 2 flasks to avoid fusion. Thus, over the 7-day period after the initial plating before implantation, passaging was carried out at day 4 and implantation at day 7.

**Cell Preparation for Implantation**

On the day of transplantation and after echocardiographic baseline functional evaluation had been performed, the cells were harvested by trypsinization and washed, and the viability was assessed with trypan blue (0.4% GIBCO BRL). A sample was plaeted onto 12-well dishes in 0.2 mL culture medium to be counted (cf infra). The cells were washed in the injection medium (culture medium + BSA 0.5%, Fraction V, Sigma Chemical Co) and kept on ice before transplantation. This process was initiated by another operator while an inferior midline ministrernotomy was carried out in the recipient rat. The cells were then pelleted, suspended in 150 μL of injection medium, and intramyocardially delivered in the infarcted area.

**Muscle Mincing Methodology**

The day of the transplantation, the left tibialis anterior muscle was harvested and trimmed of tendon, fat, and aponeurotic tissues. After rinse in F12 medium and weighing (mean weight of 500 mg), the muscular tissue was dissected, minced into a fine slurry, and suspended in a 50-μL vial (Falcon) containing 25 mL of F12 medium. The mechanical dissociation was then achieved by means of a rotative homogenizer (Polytron 89/PTRA 20TS, Kinematica GmbH LITTAU) and filtered by successive passages through decreasing diameter needles. The preparation thus obtained was temporarily injected in a 0.15-mL volume as a suspension in the infarcted myocardium scar area. One small fraction was kept for viability assessment by growth in culture medium.

**Experimental Groups**

Sixty-six rats were included in the study and were divided into 3 groups: control, mincing, and myoblast. All rats were reoperated on 1 week after myocardial infarction, under general anesthesia and tracheal ventilation, through an inferior midline ministrernotomy. After liberation of adhesions, all rats received a 150-μL injection medium delivered in the infarcted area by means of a 30-gauge needle. In the control group (n = 23), the rats received the injection medium alone. The myoblast group (n = 21) was injected with the myogenic cultured cell suspension. The mincing group (n = 22) was injected with the slurry prepared according to the protocol described above. A flow chart of the protocol is depicted in Figure 1.

**Immunochemoinal and Histochemical Studies**

**Cellular Count at Time of Transplantation**

From 18 to 24 hours after plating on the 12-well dishes, the cells were washed and fixed in methanol cooled at ~20°C for 5 minutes. A mixture (“blocking serum”) of 5% horse serum and 5% FBS in PBS solution was used to neutralize nonspecific labeling for 20 minutes. The cells were incubated with desmin mouse anti-human antibody (1:200, DAKO, A/S-Denmark) during 1 hour and next, after several washes, incubated with Cy₃-conjugated anti-mouse antibody (1:200, Jackson Immuno Research Laboratories, Inc) for 1 hour in darkness. The cells were studied under phase contrast and fluorescent illumination with the use of an inverted microscope (Olympus Optical Co, LTD). Photographs of several fields were then randomly taken. The proportion of myoblasts was calculated by dividing the
number of desmin-positive cells counted on immunofluorescent pictures by the total number of cells counted on phase contrast pictures.

**Autopsy**
Within 3 days of the last echocardiography (ie, 2 months after transplantation), hearts were removed from rats given an overdose of ketamine and xylazine. After rapid rinse in PBS, the ventricles were isolated by trimming away the atria and the valves and were cross-sectioned at the midpoint of the long axis. Both parts were frozen in isopentane cooled with nitrogen. Eight-micron-thick sections were prepared with the use of a cryostat, and standard histological studies were carried out with hematoxylin and eosin staining. For immunohistological studies, the slides were rinsed in PBS, fixed with cold methanol for 5 minutes, and the nonspecific labeling was neutralized with the blocking serum. They were incubated with the primary antibody for 1 hour and, after several washes, with the Cy™ 3-conjugated immunoglobulin antibodies. The slides were mounted in PBS/glycerol (1:1). The transplanted myoblasts were detected with the mouse monoclonal antibody directed against the embryonic myosin heavy chain (pure; kind gift of Dr Gillian Buttler-Browne, Paris, France) or against the fast skeletal myosin heavy chain (1:400, clone My 32, Sigma Chemical Co). The cardiac tissue was localized with the mouse monoclonal antibody directed against the rat cardiac α-myosin heavy chain (1:1000, clone BA-G5; kind gift of Dr Schiaffino, Padova, Italy).

**Statistical Analysis**
All data are reported as mean±1 SEM. All analysis were performed with an appropriate software (StatView, SAS Institute Inc). The critical α-level for these analyses was set at $P<0.05$.

Comparisons of continuous variables among control, mincing, and myoblast groups were studied by 1-way ANOVA followed by a post hoc test (Scheffé). If the $F$ ratio from the ANOVA and the differences detected with the Scheffé’s test were significant, a Bonferroni correction was used to further specify pairwise differences. Longitudinal studies comparing echocardiographic data within each group before and 1 and 2 months after intramyocardial injections were achieved with paired $t$ tests. Comparisons between preconditioned and nonconditioned muscles for specific myoblast cultures were assessed by 1-way ANOVA followed by a Scheffé’s test to specify differences between groups. In addition, intrarater variability in echocardiographic assessment was assessed from 2 sets of baseline measurements in 10 randomly selected rats with the use of Bland and Altman analysis.²

**Results**

**Muscle Preconditioning**
The efficacy of skeletal muscle preconditioning by bupivacaine 48 hours before harvest was demonstrated by a significant increase in the number of available myoblasts compared with nonconditioned controls (1.683×10⁶±281.120 versus 8.5×10⁵, $F=32.2, P=0.0013$).

**Characterization of Injection Suspension**
About 10 000 cells were counted on the well dishes plated on the day of transplantation, among which ≈50% were desmin positive (Figure 2). The number of injected myoblasts was $3.5×10⁵±4.6×10⁴$, ranging from $6.8×10⁴$ to $6.5×10⁵$.

**Functional Assessment**
Baseline echocardiographic parameters were not significantly different among the 3 groups. Thus, LVEF was $32.6±1.8$ in controls, $30.3±1.7$ in the myoblast group, and $36.5±2.2$ in the mincing group ($P=0.087$). In contrast, the patterns of changes after transplantation were markedly divergent. Thus, in the myoblast group, heart function improved, as assessed by LVEF, compared with the mincing and control groups.

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**Figure 1.** Protocol over 2-month period. MI indicates myocardial infarction; SM, skeletal myoblast; and Tx, transplantation.

**Figure 2.** Micrographs of primary muscle cells. Top, Whole cells under phase contrast examination. Bottom, immunofluorescence staining of desmin expression by myogenic cells in the same field (original magnification ×200).
Significant differences were detected between the myoblast and control groups at 1 month after myocardial injection (37.52 ±1.92% versus 25.49 ±2.47%, P = 0.0005). This result was sustained at 2 months after myocardial injection (40.92 ±2.17% versus 25.83 ±2.39%, P <0.0001). Differences were also noticed between the myoblast and mincing groups, but only at 2 months after transplantation (40.92 ±2.17% versus 28.57 ±2.26%, P = 0.0004). This improvement in LVEF in the myoblast group was primarily related to a smaller increase in LVESV as ventricular dilation, reflected by LVEDV, was increased to a similar extent in the 3 groups (Figure 4).

When data were analyzed within each group over the 2-month study period, a substantial improvement of LV function was found in the myoblast group (Figure 3). Differences reached statistical significance when 1- and 2-month LVEFs were compared with baseline LVEF (37.52 ±1.92% and 40.92 ±2.17% versus 30.34 ±1.74%, P = 0.0006 and P = 0.0008, respectively). The difference was also significant between 1-month and 2-month LVEF (P = 0.0069). Whereas LVEDV and LVESV increased at 1 month compared with baseline values (P<0.0001 and P = 0.0003, respectively) and 2 months (P<0.0001 and P = 0.029, respectively), a stabilization or decrease was shown thereafter when 2-month data were compared with those recorded at 1 month (P = 0.78 and P = 0.12, respectively).

The mincing and control groups featured distinct patterns. A progressive decrease in LVEF occurred in the mincing group, with differences reaching statistical significance at 2 months (P = 0.015 versus baseline). Concomitant ventricular dilatation was observed with gradual worsening from 1 to 2 months (P = 0.0017 for LVEDV). In the control group, a dramatic decrease in LVEF along with a significant increase in LVEDV were already apparent at 1 month (P = 0.0066 and P <0.0001 versus baseline, respectively). Both parameters showed the same values at 2 months.

**Immunohistology**

The presence of skeletal myofibers in the myocardium transplanted with myoblasts was assessed throughout the infarcted area by positive immunostaining for 2 different markers of skeletal muscle: embryonic myosin heavy chain and fast skeletal myosin heavy chain. The scar tissue was surrounded by cardiac muscle fibers revealed by rat cardiac α-myosin heavy chain staining (Figure 5).

**Discussion**

The two salient findings of the present study are that (1) muscle preconditioning with bupivacaine significantly increases the baseline cell yield, and (2) this increase is not sufficient to allow bypassing the phase of in vitro cell expansion and solely relying on immediate transplantation of extemporaneously minced muscle.

In vivo functional assessment was performed by 2D echocardiography, with a numeric machine allowing recording of >20 heart scans at a rate of 400 bpm (Figure 6). Rat ventricular geometry has been previously studied, and necropsy findings have demonstrated the elliptical shape of rat ventricles, which justifies our volume estimation based on an
elliptical model. We used the single-plane area-length method because 2D measurements are more appropriate than those performed in the M-mode, as shown in human infarcted hearts. The single-plane model for volume calculation was chosen in preference to the biplane method, which has not been yet validated in rat myocardial infarction and is difficult to perform because of ventricular remodeling and heterogeneous cavity dilation after large anterior infarction.

Under normal conditions, satellite cells make up 1% to 4.5% of the total number of nuclei in adult mammal muscles, depending on their fast or slow twitch fiber pattern. However, when the muscle is traumatically injured, they proliferate, migrate through and alongside basal lamina, and fuse to form new myotubes, which substitute for damaged fibers. Several pharmacological stressors can mimic trauma injury and induce a degeneration-regeneration cycle, which ultimately leads to an increased number of muscular cells. Moreover, bupivacaine potential has been demonstrated in primates, and, in murine models, the maximum activation of satellite cells occurred 2 days after muscle damage. We thus performed tibialis anterior preconditioning, using intramuscular injections 48 hours before harvest. Nevertheless, the amount of myoblast cells, obtained in these conditions, has never been assessed in the past. The hypothesis pertaining to the efficacy of bupivacaine intramuscular injections is fully supported by our data showing that the total count of myoblasts in the preconditioned muscles was almost 20-fold greater than in the contralateral untreated muscles. Although the local anesthetic bupivacaine is an attractive compound because of its availability for clinical use, some concerns have been expressed about its potential for exerting negative inotropic and/or proarythmic effects. Kinetic studies of bupivacaine in the cardiopulmonary system demonstrated high cardiac first-pass extraction of this compound, probably because of its high degree of protein binding, with increased vascular resistance of coronary artery vessels, which may affect myocardial perfusion under certain circumstances. Thus, additional studies are warranted to assess whether these putative systemic drawbacks do not offset the local drug benefits with regard to enhanced myoblast proliferation.

Having established that muscle preconditioning could successfully increase the baseline number of satellite cells, our next objective was to assess whether this pool of cells would be quantitatively sufficient to avoid in vitro cultures and proceed directly to immediate transplantation of minced muscle. The rationale behind this approach was that cultures still represent a time-consuming, logistically complex and expensive technique that is further fraught with the risk of infectious contamination at each step of the expansion process. Conversely, mincing appears as a simpler approach, which, in a clinical perspective, can be achieved expeditiously in the back table at the time of graft harvest, as demonstrated by the successful intracerebral transplantation of minced fetal brain tissue in patients with Parkinson’s disease. Furthermore, this technique addresses most of the regulatory issues raised by ex vivo manipulations of human cells. Indeed, our data clearly establish the feasibility of rapidly transforming the harvested muscle specimen into a fine slurry, which was then easily injected into the infarcted myocardium. Of note, this slurry was obtained through gentle homogenization, which did not compromise cell survival, as demonstrated by the ability of nongrafted aliquots of minced muscle to be successfully expanded in vitro (data not shown).

In addition to simplicity, a theoretically attractive feature of mincing was to allow transplantation of myoblasts along with their normal environmental matrix. This was thought to increase the likelihood of graft survival, whereas cotransplantation of other cell types, in particular fibroblasts and endothelial cells, raised the hope of increased functional benefits through limitation of scar expansion and increased angiogenesis, respectively.

Unfortunately, these expectations were not met by the results. As shown in Figure 3, hearts transplanted with minced muscle incurred a deterioration of function whose ultimate extent was similar to that seen in controls. The procedure only altered the time course of this deterioration, as it occurred slightly later than in untreated hearts. In contrast, transplantation of cultured myoblasts dramatically improved LVEF both at 1 and 2 months after cell grafting, thereby strengthening the concept, previously established by our group and others that skeletal myoblast transplantation is an effective means of ameliorating the function of infarcted myocardium. Although the mechanism of this improvement remains elusive, our observation that left ventricular remodeling was grossly similar in the 3 groups suggests that
myoblasts were primarily effective through their contractile properties, although their ability to release growth and/or angiogenic factors represents an additional possible mechanism that still must be investigated.

The reason that mincing failed remains uncertain. At least 2 hypotheses, which are not mutually exclusive, can be raised. The first is that although increased by bupivacaine preconditioning, the number of intramyocardially injected myoblasts was still too low to be functionally effective. This hypothesis is supported by recent unpublished data of our laboratory showing that posttransplantation functional outcome is linearly related to the number of injected myoblasts. A second hypothesis is that intramyocardial grafting of these multiple “chunks” of minced tissue induced a massive inflammatory response responsible for a high cell death rate. Such an attrition process caused by needle injections themselves has been previously reported by Guérette and coworkers, and the nonuse, in the present study, of anti-inflammatory drugs could have facilitated killing of grafted myoblasts by host inflammatory cells. This assumption is consistent with the delayed time course of functional deterioration yielded by our mince-transplanted hearts.

Whatever the reason that mincing was unsuccessful, our data show that in vitro cultures remain mandatory for skeletal myoblast transplantation to be functionally effective. However, the present results also suggest that it is worth exploring the possibilities of an appropriate pharmacological preconditioning of the muscle to be grafted in an attempt to increase the baseline myoblast cell yield and thus to optimize the efficacy of scale-up expansion techniques implemented during the subsequent period of in vitro cultures.

References


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