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Combined transmyocardial revascularization and cell-based angiogenic gene therapy increases transplanted cell survival

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Spiegelstein D, Kim C, Zhang Y, Li G, Weisel RD, Li RK, Yau TM. Combined transmyocardial revascularization and cell-based angiogenic gene therapy increases transplanted cell survival. *Am J Physiol Heart Circ Physiol* 293: H3311–H3316, 2007. First published September 7, 2007; doi:10.1152/ajpheart.00178.2007.—We hypothesized that pretreatment of an infarcted heart by mechanical transmyocardial revascularization (TMR) before transplantation of bone marrow cells (BMCs) or BMC-expressing angiogenic growth factors would increase transplanted BMC survival and enhance myocardial repair. Female Lewis rats underwent coronary ligation 3 wk before creation of 10 needle TMR channels (3 groups) or no TMR (3 groups), followed by transplantation of 3×10^6 male donor BMCs, BMC transfected with vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and insulin-like growth factor-1 (IGF-1) (BMC + VBI), or medium alone. At 1, 3, and 7 days, we evaluated transplanted cell survival, vascular densities, and left ventricular (LV) function ($N = 4$ per group $\times$ 6 groups $\times$ 3 time points). At 3 days, vascular densities in the scar were increased by TMR + BMC + VBI and by BMC + VBI ($P < 0.05$), and at 7 days, vascular densities were greatest in rats receiving TMR + BMC + VBI ($P < 0.05$). Transplanted cell survival at 3 and 7 days was increased by TMR and by BMC + VBI. Combined therapy with TMR + BMC + VBI resulted in the greatest cell survival at 3 days ($P < 0.05$) versus BMC. After 7 days, LV ejection fraction (LVEF) was lowest in rats receiving neither BMC nor TMR and greatest in rats receiving TMR + BMC + VBI ($P = 0.004$). We concluded that mechanical pretreatment of infarcted myocardium by TMR enhances the effect of subsequent cell-based gene therapy on transplanted cell survival, angiogenesis, and LV function. Scar pretreatment with TMR combined with cell-based multigene therapy may maximize myocardial repair.

cell transplantation; angiogenesis

TRANSPLANTATION OF VARIOUS cell types induces angiogenesis and myogenesis and improves left ventricular (LV) function in infarcted hearts. The effects of cell transplantation can be enhanced by ex vivo transfection of these cells with various transgenes. We and others have previously reported the angiogenic effect of transplantation of cells transfected with vascular endothelial growth factor sub-type 165 ($VEGF_{165}$) (19, 23, 26–28). We also noted that the simultaneous expression of VEGF, insulin-like growth factor-1 (IGF-1), and basic fibroblast growth factor (bFGF) transgenes in transplanted bone marrow cells (BMCs) had a synergistic effect on myogenesis, further enhancing transplanted cell survival, LV ejection fraction (LVEF), α- and β-myosin heavy chain, myosin light chain, and troponin I content (24, 25).

Transmyocardial revascularization (TMR) is a therapy intended to enhance myocardial perfusion through the creation of channels, either by laser or mechanical means, in ischemic myocardium (8, 13). Clinical studies have reported angina relief in many patients following laser TMR (5, 7), although there is still controversy over how TMR acts in humans. In animals, however, TMR, by either mechanical or laser injury, is known to upregulate VEGF, bFGF, and other growth factors and induce at least a limited degree of angiogenesis (2, 6). Most studies have concluded that similar responses can be elicited by mechanical versus laser TMR (3, 4, 14), although at least one study has documented greater angiogenesis with laser versus mechanical TMR (12).

In the current study, we hypothesized that the limited angiogenesis noted after mechanical TMR of a large infarct might be due to inadequate numbers of the required endothelial cells and smooth muscle cells. Cell transplantation may enhance angiogenesis after pretreatment of the infarcted myocardium with mechanical TMR by supplying these cellular components. Conversely, the efficacy of cell transplantation is limited by early cell loss. We hypothesized that TMR might increase survival of transplanted cells and thus their angiogenic and myogenic potential. TMR and cell transplantation may thus complement the limitations of each other and have synergistic effects on angiogenesis and myogenesis.

MATERIALS AND METHODS

Animals and experimental model. All experimental procedures were approved by the Animal Care Committee of the University Health Network and were performed according to the *Guide for the Care and Use of Laboratory Animals*, prepared by the National Research Council.

Adult female and male syngeneic Lewis rats were obtained from Charles River Canada (Quebec, QC, Canada). Female rats weighing 200–250 g served as recipients, and male rats weighing 250–300 g were used as cell donors.

Myocardial infarctions were induced in female Lewis rats by ligation of the left anterior descending (LAD) coronary artery. Briefly, rats were anesthetized with ketamine hydrochloride (20 mg/kg) and pentobarbital sodium (30 mg/kg), intubated, and ventilated with room air supplemented with oxygen and isoflurane (0.5%-2%) by a Harvard ventilator. Through a left lateral thoracotomy, a 6-0 polypropylene suture was used to ligate the proximal LAD coronary artery. The incision was closed, and antibiotics (150,000 U/ml penicillin G benzathine and 150,000 U/ml penicillin G procaine) and buprenorphine hydrochloride (0.01 mg/kg) were administered after operation.

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BMC isolation. Male donor BMCs were isolated from rat femurs and cultured in Iscove's modified Dulbecco's medium with 10% FBS for 7 to 10 days before harvesting (at 60% to 70% confluence). The cultures were partially depleted of erythroid progenitor cells by the removal of nonadherent cells with each change of medium every 3 days. No additional fractionation was performed. The cells were evaluated by flow cytometry before transplantation. CD45 was expressed in 22% of the cells, whereas CD34 was expressed in only 3%, and stem cell antigen-1 (Sca-1) was expressed in 30%.

Cell transfection. Cells were transfected ex vivo with plasmids encoding $VEGF_{165}$ (pCEP4-VEGF), IGF-I (PUC18-IGF-I), and bFGF (PCDNA3.1-bFGF) (23, 27, 28). Transfection efficiencies were monitored by cotransfection with pEGFP-N2 (Clontech BD Biosciences), encoding green fluorescence protein, and were ~30%. Cells were incubated with the transfection reagents for 24 h before transplantation.

TMR and cell transplantation. Three weeks after infarct creation, rats were randomly assigned to six experimental groups. Three groups underwent mechanical TMR followed after 15 min by cell transplantation as described below. The other three groups underwent cell transplantation without prior TMR.

TMR was performed by creating 10 transmural channels with a 23-gauge needle in a 10 mm × 10 mm area within the LAD infarct. Hemostasis was obtained with local pressure.

Rats were injected with culture medium alone (2 groups, with/without TMR, referred to as TMR + medium and medium, respectively), transplanted with 3×10^6 untransfected BMCs (2 groups, with/without TMR, referred to as TMR + BMC and BMC, respectively), or transplanted with BMCs transfected with VEGF, bFGF, and IGF-I (collectively referred to as VBI) (2 groups, with/without TMR, referred to as TMR + BMC + VBI and BMC + VBI, respectively) ($N = 4$ per group × 6 groups × 3 time points = 72 recipient rats in total).

The rats were euthanized 1, 3, and 7 days after cell transplantation for histologic evaluation and for DNA isolation for polymerase chain reaction (PCR). After death, the free wall of the LV was excised and divided into two equal sections. One section was utilized for histological analysis, and the other section was utilized for real-time PCR ($n = 4$, for each group at each time point). The LV was divided into the scar zone (transmural scar), the border zone (partial-thickness scar containing both fibrous tissue and surviving muscle), and the normal myocardium.

Quantitative histology of vascular densities. In each experimental group, 50% of each rat heart was subjected to quantitative histological analysis ($n = 4$, for each group at each time point). Hearts were sectioned along their long axis, and sections were fixed in 5% glacial acetic acid in methanol, embedded in paraffin, and sectioned into 10-μm slices. Sections were stained with hematoxylin and eosin or immunohistochemical staining against factor VIII. The total number of vessels per high-power field (0.2 mm^2), regardless of size, was counted by two blinded observers in five randomly selected fields per heart. The mean number of vessels per field was used for analysis.

Quantitation of Y chromosomal DNA by real-time PCR. DNA was isolated from snap-frozen and powdered heart specimens, as described previously (9, 25, 26). To accurately quantitate the number of Y chromosome-bearing male donor cells within the female recipient hearts, a pair of specific primers was designed based on the GeneBank sequence (National Center for Biotechnology Information, Washington, DC) of the sex-determining region of the Y chromosome (sry) of the rat (sense 5′-GAGGCACAAGTTGGCTC-AACA-3′, antisense 5′-CTCCTGCAAAAAGGGCCTTT-3′). Quantitative PCR was performed on the 9700 HT System (Applied Biosystems) using the Master Mix SYBR Green Kit (Applied Biosystems), utilizing threefold serial dilutions of the sry2 DNA standards in distilled deionized water (ddH_2O) to generate standards ranging from the sry2 DNA of 1×10^4 cells to the sry2 DNA of 41 cells. Briefly, total DNA content was measured spectrophotometrically, and equal amounts of DNA were taken from all samples and were transferred to a 96-well PCR plate. Each assay was performed in duplicate, and 5 μl of ddH_2O were assayed as a no-template control. Five microliters of a 5 pmol sry2 sense and antisense primer mixture and 10 μl of Master SYBR Green Mix were added to each well. The reaction sequence included stabilization for 10 min at 50°C before 45 cycles of denaturation for 15 s at 95°C, annealing and primer extension for 1 min at 60°C, and dissociation for 15 s at 95°C, 15 s at 60°C, and 15 s at 95°C. PCR products were labeled with SYBR Green and detected by a laser detector on the 9700 HT system. Real time PCR data were analyzed with SDS 2.1 software (Applied Biosystems).

LV function. LV function was evaluated in vivo in rats by transthoracic echocardiography (ACUSON Sequoia C256; Siemens, Malvern, Pennsylvania), as described by Sahn et al. (16) and Schiller et al. (17). Left parasternal images were taken in the right lateral decubitus position with the use of a 15-MHz linear transducer. Two-dimensional mode images in the parasternal long-axis view of the LV and M-mode images at the level of papillary muscles were recorded. LV end-diastolic volume (LVEDV), LV end-systolic volume (LVESV), LV end-diastolic area (LVEDA), and LV end-systolic area (LVESA) were measured. Ejection fraction (EF), fractional area shortening (FAS), and fractional shortening (FS) were calculated with standard M-mode echocardiographic equations (EF = LVEDV − LVESV/LVEDV × 100; FAS = LVEDA − LVESA/LVEDA × 100; FS = LVEDD − LVESD/LVEDD × 100). For each measurement, three consecutive cardiac cycles were traced and averaged by an experienced examiner in a blinded fashion. All animals were measured at baseline, before treatment, and one day before death ($n = 4$ in each group at each time point).

Statistical analysis. Data are presented as means ± SD. Continuous data were analyzed by ANOVA. When the ANOVA *F*-value was significant, a post hoc Scheffé's multiple range test was used to specify the differences.

RESULTS

Histology. Histological examination of the TMR channels demonstrates needle tracts lined with endothelial cells along and around the channel (Fig. 1).

Vascular densities. One day after cell transplantation, quantitative vascular densities were similar in all six groups, in the scar, border zone, and normal myocardium. On *day 3*, vascular densities in the scar were greatest in the TMR + BMC + VBI group ($P < 0.05$ vs. BMC + VBI, TMR + BMC, BMC, TMR + medium, and medium) (Fig. 2*A*). Vascular densities in the scar of the BMC + VBI group at this time point were also higher than BMC and medium groups ($P < 0.05$). There were no differences between groups in vascular densities in the border zone or normal myocardium.

Seven days after cell transplantation, vascular densities in the scar had, in general, increased from levels noted at 3 days. Densities were again greatest in the scar of the TMR + BMC + VBI group ($P < 0.05$ vs. BMC + VBI, TMR + BMC, BMC, TMR + medium, and medium) (Fig. 2*B*). Again, there were no differences between groups in the border zone or normal myocardium.

Cell survival. One day after cell transplantation, cell survival ranged from a low of 71% in the BMC group to a high of 92% in the TMR + BMC + VBI group, although these differences did not reach statistical significance (Fig. 3).

Cell survival declined progressively in all cell-transplanted groups at *day 3* and *day 7*. However, at three days, ~70 ± 26% of the transplanted BMC in the TMR + BMC + VBI group had survived versus 57 ± 21%, 47 ± 18%, and 36 ± 26% in

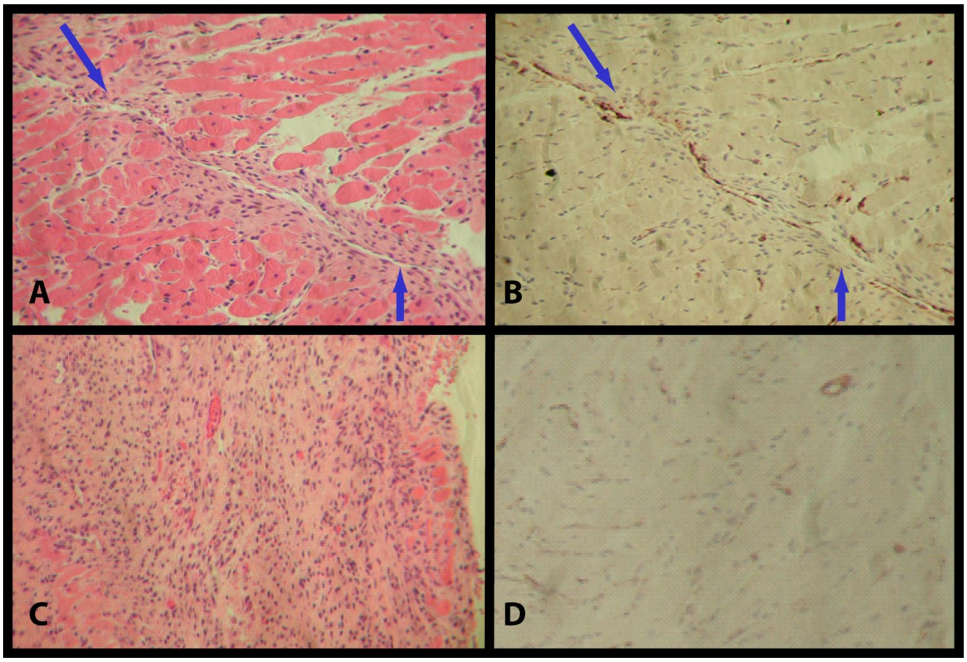

Fig. 1. Photomicrographs of rat hearts 1 wk after transmyocardial revascularization (TMR) (*A* and *B*) or no TMR (*C* and *D*). Hematoxylin and eosin staining (*A* and *C*). A TMR channel (blue arrows) in the border zone of the infarct is shown (*A* and *B*). Immunostaining for factor VIII (*B* and *D*) demonstrates endothelial cells along and around the TMR channel (*B*).

BMC + VBI, TMR + BMC, and BMC groups, respectively ($P < 0.05$ vs. BMC) (Fig. 3). At 7 days, differences between groups were slightly lower in magnitude. At this time point, ~50% of the implanted BMCs had survived in the TMR + BMC + VBI group, compared with only about 25% of unmodified BMC without TMR, although these differences did not reach statistical significance.

LV function. Ligation of the LAD coronary artery reduced LVEF from 78 ± 11% to 60 ± 3% before treatment. The magnitude of this decline was similar in all groups.

Three days after cell transplantation, the lowest LVEF was noted in the medium group, with a slight improvement in the TMR + medium and BMC groups, a further increase in the TMR + BMC group, and the greatest LVEF in the TMR + BMC + VBI and BMC + VBI groups (Fig. 4). However, these differences did not reach statistical significance.

At 1 wk, differences were more apparent. There was an obvious progression of LVEF from a minimum in the medium group to a maximum in the TMR + BMC + VBI group ($P < 0.05$). TMR alone induced a small and nonsignificant increase in LVEF at 7 days, but BMC transplantation was associated with a greater increase. The incremental effect of transfection with VEGF, bFGF, and IGF-1 appeared to be greater than the incremental effect of TMR, and there was a clear additive effect of TMR and transfected BMC.

Other echocardiographic parameters, including FAS and FS, showed similar changes over time and differences between groups.

DISCUSSION

The effects, whether structural, paracrine, or in induction of progenitor cell engraftment, of cell transplantation are almost certainly directly proportional to the number of cells engrafted. Survival of the transplanted cells is therefore crucial to maximize angiogenesis and myogenesis in the infarcted heart. We have previously reported that transfection of cells with selected transgenes before their transplantation augments cell survival, angiogenesis, and myogenesis (23–28). However, cell survival, even with this strategy, is suboptimal, and we therefore investigated the potential effects of scar pretreatment, in this case by mechanical TMR, on the survival and efficacy of the transplanted cells.

TMR by laser or mechanical means induces upregulation of angiogenic growth factors and increases perfusion and function of ischemic myocardium. Clinical trials of TMR have reported significant relief of angina (1, 7, 18), although its mechanism of action in humans is still controversial. TMR may modify the infarct zone to enhance the survival and efficacy of implanted cells by inducing angiogenesis and other effects. TMR of the scar, in addition to transfection of the cells, may augment repair of the infarcted myocardium.

In this experiment, we hypothesized that the combination of mechanical pretreatment of infarcted myocardium (by TMR) and transplantation of transfected BMC would improve cell survival and thus augment angiogenesis and LV function. We employed BMCs (which were depleted of hematopoietic precursors but were not further fractionated) as carrier cells, since they have greater plasticity after engraftment than myocytes (29) and improve LV function (20).

In our model, TMR alone without cell transplantation (the TMR + medium group) did not result in appreciable differences in vascular densities compared with the control group, which received neither TMR nor cells (medium group). However, this medium control group did receive an injection of culture medium, and this injection might have resulted in myocardial injury of a nature similar to that induced by our needle TMR but of lower magnitude. We did not have a control group that received no manipulation of the infarct of any kind. Since our study was not designed with the intent of comparing TMR with medium injection to medium injection alone, the effect of isolated TMR is difficult to evaluate. No difference was noted between the TMR + medium and the medium

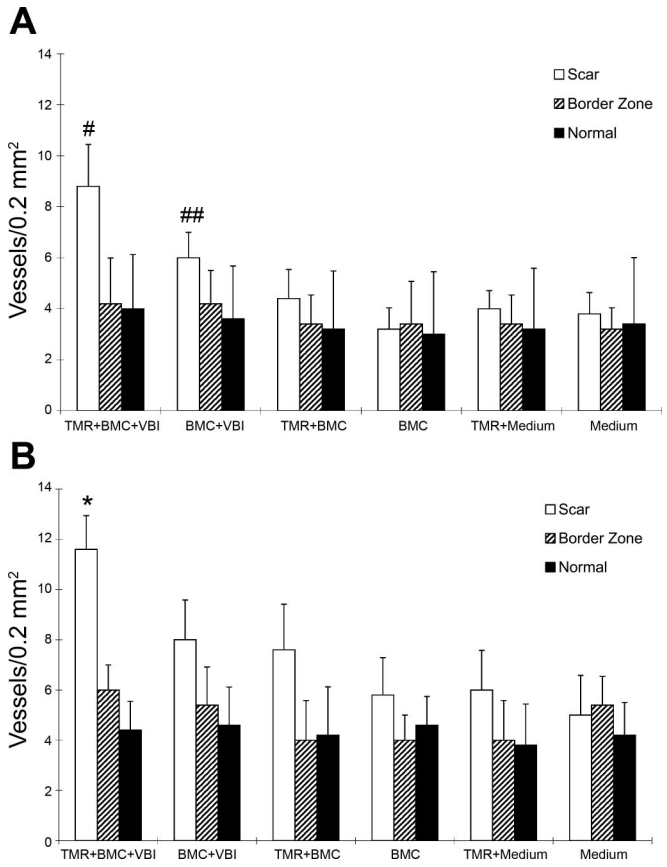

Fig. 2. Vascular densities in the scar, border zone, and normal myocardium in a chronic rat infarct 3 days (*A*) and 1 wk (*B*) after TMR or no TMR, followed by cell transplantation. Vascular densities in the scar were lowest in control rats receiving neither TMR nor cell transplantation. TMR and transplantation of unmodified bone marrow cells (BMCs) both increased vascular densities slightly but not to a statistically significant degree. The addition of either TMR or transgene expression to transplanted BMCs further increased vascular densities. Vascular densities were greatest in rat hearts pretreated with TMR before transplantation of transfected BMC ($P < 0.05$ vs. BMC + VBI, TMR + BMC, BMC, TMR + medium, and medium), where VBI represents vascular endothelial growth factor (VEGF), basic fibroblast growth factor (BFGF), and insulin-like growth factor-1 (IGF-1) collectively. There was no difference between groups in vascular densities in the border zone or the normal myocardium. #$P < 0.05$ vs. BMC + VBI, TMR + BMC, BMC, TMR + medium, and medium; ##$P < 0.05$ vs. BMC and medium; *$P < 0.05$ vs. BMC + VBI, TMR + BMC, BMC, TMR + medium, and medium.

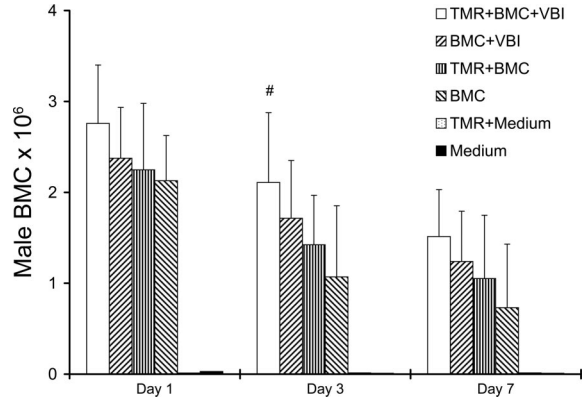

Fig. 3. The number of transplanted male BMCs measured 1, 3, and 7 days after implantation. The number of donor-derived cells was lowest in unmodified BMCs. TMR preceding BMC transplantation tended to increase donor cell numbers, particularly at 3 and 7 days. Transgene expression had a similar but slightly greater effect on implanted BMCs. The greatest cell numbers were seen when TMR preceded implantation of transfected BMCs. Whereas donor-derived cell numbers decreased in all groups with time, total male cell numbers were greatest at all time points in the TMR + BMC + VBI group. #$P < 0.05$ vs. BMC at *day 3*.

groups in vascular densities, and survival of transplanted cells was not an issue, since no cells were implanted. However, there was a small increase in LVEF in the TMR + medium group compared with the medium rats. Although this difference did not reach statistical significance, it is consistent with the rest of our data, suggesting that TMR improved function and perfusion, and with reports of Wang and colleagues (21, 22), noting a beneficial effect of TMR on LV function.

The additive effect of TMR with cell transplantation on outcomes such as vascular densities and cell survival is much more apparent. TMR increased the effect of BMC transplantation on cell survival, angiogenesis, and LV function. The effect of TMR on these outcomes when transfected BMCs were implanted was even greater.

TMR has been reported to induce angiogenesis (1, 6), and our current data support this theory; however, somewhat surprisingly, the effect of TMR on vascular densities was noted as early as 3 days after transplantation of transfected BMC. This early angiogenic response to TMR may have been potentiated by overexpression of the angiogenic transgenes by the implanted BMC.

Cell survival after implantation was quantitated by real-time PCR analysis of the Y-chromosomal gene sry2 from male donor cells in female-recipient hearts (9). The LV free wall was divided into two equal sections for histology and DNA isolation, and we are aware that this method assumes homogeneous distribution of transplanted cells in the LV wall. We noted a loss of ~30% of the cells immediately after injection, presumably because of mechanical factors, followed by a progressive decrease over time. Most studies have suggested that the majority of cell loss occurs early after implantation, implying that strategies to maximize early cell survival will ultimately result in greater late survival. We observed that cell loss after cell transplantation was reduced in the rats pretreated with TMR at all time points. Although the angiogenic effect of

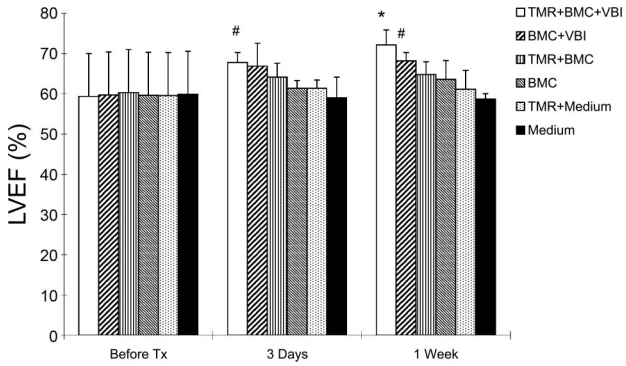

Fig. 4. Left ventricular ejection fraction (LVEF) before TMR and cell transplantation and 3 and 7 days afterward. LVEF was lowest in rats injected with culture medium alone and progressively increased through the TMR, BMC, TMR + BMC, and BMC + VBI groups. The greatest LVEF, at 3 and 7 days, was in the TMR + BMC + VBI group. #$P < 0.05$ vs. medium; *$P < 0.05$ vs. BMC, TMR + medium, and medium.

TMR has been widely reported (2, 6, 10, 11, 15), its effect on transplanted cell survival is not clear and, to our knowledge, only the survival of embryonic germ cells has been evaluated (22).

The differences and trends in cell survival between the groups are similar at all time points but reached statistical significance only in *day 3* and thus appear to be internally consistent. Some of these differences reached statistical significance at *day 3* but not at *day 7*. Given the consistency in the data trends, this lack of statistical significance is likely related to insufficient power. We believe that had it been possible to utilize a larger number of rats for this study, we would have noted lower variances in our data and thus achieved statistically significant differences in more of these outcome measures.

The mechanisms by which TMR performed only 15 min before cell transplantation enhanced BMC survival are still unclear, since it is probable that the angiogenic and other beneficial effects of TMR require several days or weeks to develop. One potential mechanism may be enhanced perfusion of the implanted cells. Although channels induced either by needle or laser TMR are generally thought to occlude within the first week or two, it is possible that greater perfusion of the infarct zone in the first several days, through the needle channels before their eventual closure, may have resulted in greater survival of the implanted BMCs. There may be a second phase in which new blood vessel development induced by TMR results in improved perfusion, but this is unlikely to play a role in determining cell survival at 1 day. As with any form of myocardial injury, TMR induces a local inflammatory response, and this inflammatory response might lead to enhanced local release of prosurvival cytokines. However, clarification of the mechanism(s) responsible for improved survival of the transplanted cells will require further investigation.

Our study has several limitations. Although we observed improvement in vascular density, cell survival, and LV function at 1 wk, we did not assess the durability of those effects in the medium or long-term period. In our study, we have not assessed growth factor expression, which may explain the early angiogenic response, as was observed. Further studies and studies in which laser TMR is performed in larger animals will be required to determine whether the effect noted here is generalizable to other modalities of TMR and whether it is durable. Our study does not permit us to comment on the optimal interval between TMR and subsequent cell transplantation. It is to some degree unexpected that an interval of as little as 15 min could still result in a beneficial effect of TMR. It is entirely possible that longer intervals may allow greater effects of TMR. In this study we used BMC as carrier cells, as they have greater plasticity after engraftment than myocytes. The selection of the optimal cell type (BMCs, heart cells, skeletal myoblasts, endothelial cells, etc.) to combine with mechanical pretreatment of scar (mechanical or laser TMR) is still unclear and needs further investigation.

In conclusion, our study demonstrated improvement of cell survival, angiogenesis, and LV function when the infarct zone is pretreated by needle TMR (mechanical TMR) as little as 15 min before cell transplantation. This combined therapy may represent an improvement to isolated cell therapies. Clinically, a patient with an extensive infarct might undergo transcatheter TMR and simultaneous BMC aspiration for expansion of BMCs ex vivo, followed after several weeks by BMC implantation and concurrent surgical or percutaneous revascularization. Future studies are needed to define the potential role of this novel combination therapy.

GRANTS

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