Preischemic autologous mitochondrial transplantation by intracoronary injection for myocardial protection

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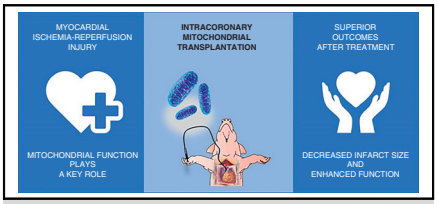
ABSTRACT

Objective: To investigate preischemic intracoronary autologous mitochondrial transplantation (MT) as a therapeutic strategy for prophylactic myocardial protection in a porcine model of regional ischemia-reperfusion injury (IRI).

Methods: The left coronary artery was cannulated in Yorkshire pigs (n = 26). Mitochondria (1×10^9) or buffer (vehicle [Veh]) were delivered as a single bolus (MT_S) or serially (10 injections over 60 minutes; MT_{SS}). At 15 minutes after injection, the heart was subjected to temporary regional ischemia (RI) by snaring the left anterior descending artery. After 30 minutes of RI, the snare was released, and the heart was reperfused for 120 minutes.

Results: Coronary blood flow (CBF) and myocardial function were increased temporarily during the pre-RI period. Following 30 minutes of RI, MT_S and MT_{SS} hearts had significantly increased CBF that persisted throughout reperfusion (Veh vs MT_S and MT_{SS}; $P = .04$). MT_S and MT_{SS} showed a significantly enhanced ejection fraction (Veh vs MT_S, $P < .001$; Veh vs MT_{SS}, $P = .04$) and developed pressure (Veh vs MT_S, $P < .001$; Veh vs MT_{SS}, $P = .03$). Regional function, assessed through segmental shortening (Veh vs MT_S, $P = .03$; Veh vs MT_{SS}, $P < .001$), fractional shortening (Veh vs MT_S, $P < .001$; Veh vs MT_{SS}, $P = .04$), and strain analysis (Veh vs MT_S, $P = .002$; Veh vs MT_{SS}, $P = .003$), was also significantly improved. Although there was no difference in the area at risk between treatment groups, infarct size was significantly reduced in both MT groups (Veh vs MT_S and MT_{SS}, $P < .001$).

Conclusions: Preischemic MT by single or serial intracoronary injections provides prophylactic myocardial protection from IRI, significantly decreasing infarct size and enhancing global and regional function. (J Thorac Cardiovasc Surg 2020;160:e15-29)



Mitochondrial transplantation protects against myocardial ischemia-reperfusion injury.

Central Message

Preischemic autologous mitochondrial transplantation by intracoronary injection is a novel strategy that provides prophylactic myocardial protection from ischemia-reperfusion injury.

Perspective

We present the results of preischemic autologous mitochondrial transplantation by intracoronary injection for prophylactic myocardial protection in a model of regional ischemia-reperfusion injury. This new technique may provide a novel prophylactic methodology to reduce morbidity and mortality in patients at risk of ischemia-reperfusion injury.

See Commentaries on pages e31 and e33.

Coronary artery disease is the leading cause of death and disability worldwide, with more than 9 million attributed deaths in 2016.[1] There are sufficient data to show that ischemia-reperfusion injury (IRI) leads to alterations in cellular energy and homeostasis, accumulation of reactive oxygen species, and DNA damage, all of which converge on mitochondria.[2-4] This ultimately activates signaling for apoptosis and necrosis and presents as myocardial dysfunction.[5]

To address IRI, we pioneered a novel therapy, termed autologous mitochondrial transplantation (MT), consisting of the functional recovery of native, damaged mitochondria with viable mitochondria isolated from the patient's own healthy skeletal muscle.[6-19] Viable, respiration-competent mitochondria are isolated in a procedure taking less than 30 minutes,[8] allowing for application during the same

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Abbreviations and Acronyms

AAR = area at risk
CBF = coronary blood flow
HR = heart rate
IRI = ischemia-reperfusion injury
LAD = left anterior descending artery
LV = left ventricular
LVEF = left ventricular ejection fraction
MAP = mean arterial pressure
MT = mitochondrial transplantation
Pdev = peak developed pressure
Ped = end-diastolic pressure
dP/dt max = maximal change in pressure over time
RI = regional ischemia
Veh = vehicle

operation or at the patient's bedside, with single or multiple doses.[8]

We have previously demonstrated that both direct injection and intravascular coronary injection MT during early reperfusion is safe and effective, significantly decreasing myocardial infarct size and enhancing postischemic functional recovery.[10] In this study, we investigated for the first time the use of single or serial intracoronary MT before ischemia, hypothesizing that preischemic MT would provide prophylactic myocardial protection.

METHODS

Animal Care and Bio Safety

This investigation was conducted in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and was approved by the Boston Children's Hospital's Animal Care and Use Committee (protocol 16-04-3169). All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals.

Experimental Model

Female Yorkshire pigs (n = 26, 40-60 kg) were selected at random to receive vehicle (Veh; n = 10), single MT injection (MT_S; n = 10), or serial MT injections (MT_{SS}; n = 6). The animals were sedated with intramuscular Telazol (2.2-4.4 mg/kg) and xylazine (1-2 mg/kg). Endotracheal intubation was performed, and general anesthesia was induced with isoflurane (3% induction, 0.5%-2.0% maintenance). Ventilatory frequency and volumes were adjusted to maintain physiological arterial blood gas values. Normothermia was maintained using a water-perfused heater pad. Femoral lines were placed in a sterile fashion for continuous mean arterial pressure (MAP) and central venous pressure monitoring. Intravenous heparin 100 U/kg and 2% lidocaine 2 mg/kg were injected at the start of the procedure.

Following verification of deep anesthesia, sternotomy was performed. The pericardium was opened, the left anterior descending artery (LAD) was dissected distal to the second diagonal branch, and a perivascular flow probe (Transonic Systems, Ithaca, NY) was placed circumferentially. Coronary blood flow (CBF) was continuously recorded through a T403 Multichannel Research Console (Transonic Systems) and analyzed using LabChart 7 acquisition software (AD Instruments, Sydney, Australia).

A suture was passed around the LAD, and both ends were passed through a small vinyl tourniquet to form a snare. The right carotid artery was then cannulated with a 6F angiography sheath using a direct cut with exposure of the vessels. Selective catheterization of the left coronary artery was performed using a 5F multipurpose guide catheter (Merit Medical Systems, South Jordan, Utah), followed by injection of iodinated contrast medium (Optiray 350 [ioversol 74%]; Guerbet, Villepinte, France) (Figure 1; Video 1).

Mitochondrial Isolation

The pectoralis major was located and dissected, and a small piece was surgically extracted using a 6-mm biopsy punch (approximately 0.01 g) (Miltex, York, Pa) and used for mitochondrial isolation, as described previously.[8] The isolated mitochondria were suspended in vehicle solution (250 mM sucrose, 10 mM K^+-Hepes pH 7.2, and 0.5 mM K^+-EGTA pH 8.0).[13]

Experimental Groups

Animals treated by intracoronary injection were divided into 3 groups: those receiving either vehicle solution alone (Veh; 6 mL) or vehicle solution containing MT, either single injection (MT_S) or serial injections (MT_{SS}). Single injections were delivered as a bolus antegrade into the left main coronary artery (1×10^9 in 6 mL; n = 10). Serial injections (10 injections of 1×10^9 in 6 mL of respiration buffer in each injection; n = 6) were delivered every 5 minutes (Figure 2).

The hearts were allowed to recover for 15 minutes after the final injection. Temporary regional ischemia (RI) was induced by snaring the LAD. After 30 minutes of RI, the snare was released, and then the heart was reperfused for 120 minutes. Angiography was performed to confirm LAD patency (Figure 1; Video 1).

Left Ventricular Global and Regional Function

Global left ventricular (LV) function was evaluated with a 7F pressure-volume conductance catheter (Transonic Systems) inserted through the apex. Data were continuously recorded using LabChart 7 Acquisition Software (AD Instruments). LV peak developed pressure (Pdev, in mm Hg), LV end-diastolic pressure (Ped, in mm Hg), and maximal change in LV pressure over time (dP/dt max, in mm Hg/s) were obtained.

Echocardiography was performed using a Philips iE33 machine with a 5-MHz transducer (Philips Healthcare, Amsterdam, The Netherlands). Two-dimensional echocardiography, M-mode echocardiography with 2-dimensional guidance, and Doppler echocardiography were used to measure the size and volume of the LV cavity. Images and data were obtained as recommended by the American Society of Echocardiography Standards for assessment of LV function.[20]

Regional myocardial function was assessed by sonomicrometry (Sonometrics Digital Ultrasonic Measurement System, Sonometrics, London, ON, Canada), echocardiography, and endocardial global circumferential strain. Four digital piezoelectric ultrasonic probes (2.0 mm) were placed in the subendocardium in the area of RI. Digital data were inspected using postprocessing software (SonoView; Sonometrics). Regional echocardiographic measurements were obtained on epicardial short-axis images, aligning the cursor just below the mitral leaflets, in the area of RI. Strain analysis was performed offline with TomTec 2D Cardiac Performance Analysis (TomTec Imaging Systems, Munich, Germany). Because endocardial global circumferential strain represents fiber shortening, this is expressed as a negative numeric value, with a greater negative value representing greater shortening.[21]

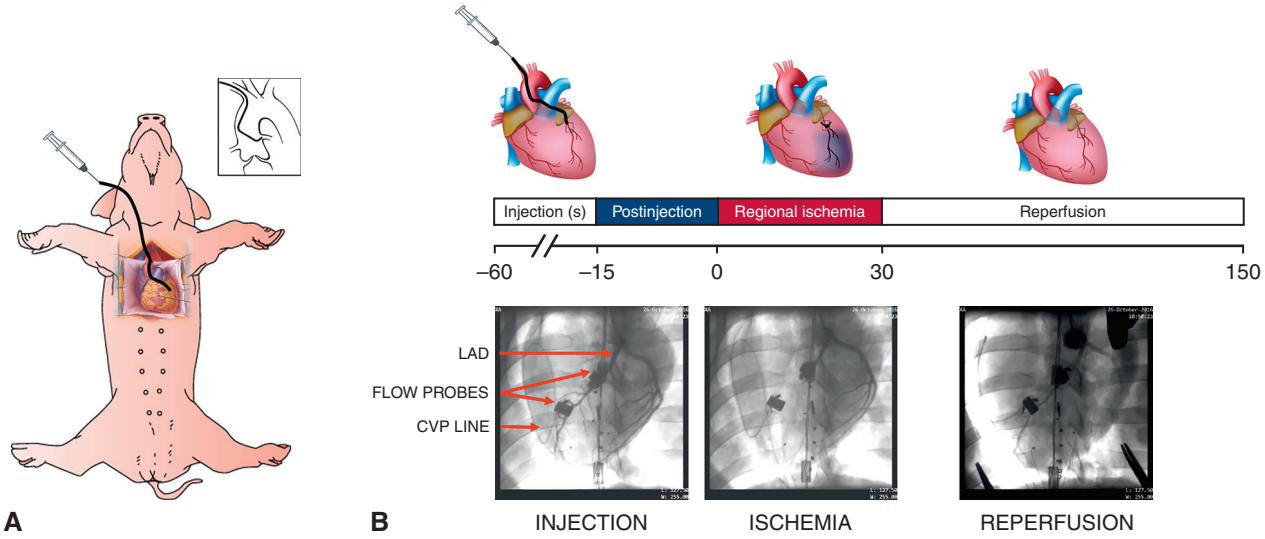


FIGURE 1. Description of experimental model. A, Female Yorkshire pigs (40-50 kg) were sedated and intubated. Sternotomy was performed, and the pectoralis major was located and dissected, 2 small pieces of which were excised using a 6-mm biopsy punch for mitochondrial isolation. B, Vehicle or mitochondria resuspended in vehicle were delivered as a 6-mL bolus, antegrade to the left main coronary under fluoroscopic guidance using a 5F Judkins right angiography catheter. At 15 minutes after the end of injection, regional ischemia was achieved by temporarily snaring the left anterior descending artery (*LAD*) after the second diagonal branch. After 30 minutes of ischemia, the snare was released, and the heart was reperfused for 120 minutes. Angiography was then performed to assess LAD patency and heart reperfusion. *CVP*, Central venous pressure.

Euthanasia

After 120 minutes of reperfusion, the heart was removed and the animal euthanized by exsanguination in accordance with the American Physiological Society's Guiding Principles for the Care and Use of Vertebrate Animals in Research and Training protocol. After euthanasia, all hearts were harvested for histological analysis, imaging, and wet/dry weight measurements.

Area at Risk/Infarct Size

The ischemic area at risk (AAR) was delineated by LAD ligation, cross-clamping of the aorta, and subsequent injection of blue monocrystalline pigment (diluted 1:5 in PBS) into the aortic root.[22] The heart was then removed, and the left ventricle was partitioned along the long axis, from apex to base, into 1-cm-thick transverse sections. The AAR was traced onto a clear acetate sheet over a glass plate under room light, after which infarct size was determined with triphenyl tetrazolium chloride as described previously.[6,7] Infarct size was determined by a blinded observer. Wet/dry weight was determined as described previously.[6,7]

Histology and Transmission Electron Microscopy

LV samples from the AAR were collected for histology and transmission electron microscopy as described previously.[6,7,10] Hematoxylin and eosin–stained slides were evaluated for necrosis and inflammatory cells infiltration. All histological and electron microscopy was performed by a blinded observer.

Statistical Analysis

Continuous variables are expressed as mean ± standard error. The normality of all continuous variables was tested using the Shapiro–Wilk test and graphically assessed by histograms and Q-Q plots. Longitudinal analysis for between-group comparisons was performed using 2-way repeated-measures analysis of variance (ANOVA) and by fitting mixed-effects linear regression models. When a significant F-test was obtained on overall 2-way repeated-measures ANOVA, a Bonferroni-adjusted post hoc analysis was used to assess pairwise differences between groups. One-way ANOVA was used for between-group comparisons in the case of histopathological indices. To reduce the probability of false-positive results (type I error) due to the multiple comparisons, the Benjamini–Hochberg false discovery rate (FDR) was applied to control familywise error to $\alpha < 0.05$. All reported tests are 2-tailed. Statistical analyses were performed using Stata version 15.1 (StataCorp, College Station, Tex) and GraphPad Prism version 7.00 for Mac OS X (GraphPad Software, La Jolla, Calif).

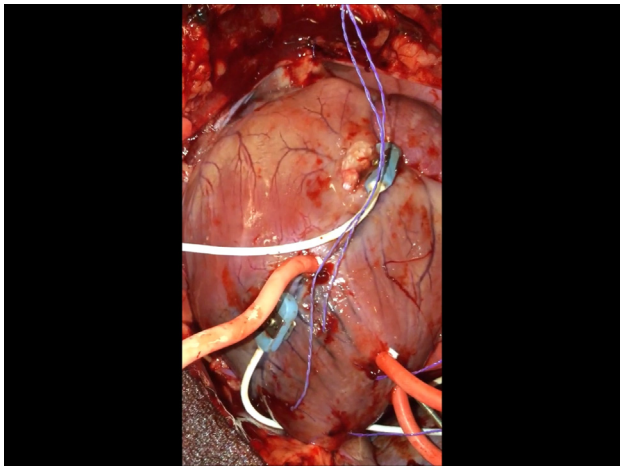
RESULTS

Myocardial Function

PV loop analysis, sonomicrometry, and echocardiographic assessment of heart function (both global and regional) did not reveal any difference between Veh and MT_S or MT_{SS} groups before injection ($P > .05$ for each) (Figures 3 and 4; Table E1).

Both MT_S and MT_{SS} induced a transient increase in CBF significantly different from that seen with Veh for up to 5 minutes (Figure 3, *A*). This increase in CBF was consistent and reproducible for all injections in the MT_{SS} group (Figure E1). MT_S and MT_{SS} had no effect on heart rate (HR) or MAP (Figure E2).

Intracoronary delivery of mitochondria significantly increased LV function temporarily. LV Pdev was increased



VIDEO 1. Video showing the experimental model. Female Yorkshire pigs were sedated and intubated. A sternotomy was performed, and the pectoralis major was located and dissected. Vehicle or mitochondria resuspended in vehicle were delivered as a 6-mL bolus antegrade to the left main coronary under fluoroscopic guidance. At 15 minutes after the end of injection, regional ischemia was achieved by temporarily snaring the left anterior descending artery (LAD) after the second diagonal branch. After 30 minutes of ischemia, the snare was released, and the heart was reperfused for 120 minutes. Angiography was then performed to assess LAD patency and heart reperfusion. Video available at: https://www.jtcvs.org/article/S0022-5223(19)31663-0/fulltext.

for 5 minutes after MT, whereas LV ejection fraction (LVEF) and dP/dt max were still enhanced at the end of the 15-minute period preceding RI (Figure 3, *B* and *C*). At the end of this period before RI, no significant difference was observed for regional function between the groups (Figure 5).

RI

CBF and LV function were significantly decreased in the MT_S, MT_{SS}, and Veh groups during RI compared with the end of the intracoronary injection period (Figures 4 and 5; Table E2). LV Pdev was significantly higher in the MT_S and MT_{SS} groups compared with the Veh group ($P = .04$ and $P < .004$, respectively) (Figure 4, *B*). Electrocardiogram changes related to ischemia were similar in all groups.

Postischemia Comparison: Global Function After MT and a Subsequent Ischemic Event

After 120 minutes of reperfusion, significantly increased LV global function was seen in the MT_S and MT_{SS} groups (Table E1). The LVEF was 36.1 ± 2.1% in the Veh group, 53.6 ± 2.9% in the MT_S group ($P < .001$ vs Veh), and 50.3 ± 4.3% in the MT_{SS} group ($P = .04$ vs Veh) (Figure 4, *A*).

After 120 minutes of reperfusion, LV Pdev was significantly increased to 74.9 ± 2.6 mm Hg in the MT_S group and 69.9 ± 3.7 mm Hg in the MT_{SS} group, compared with 57.8 ± 2.4 mm Hg in the Veh group ($P < .001$ and $P = .03$ vs Veh, respectively) (Figure 4, *B*).

Similarly, LV dP/dt max after 120 minutes of reperfusion was significantly increased to 988 ± 69 mm Hg/s in the MT_S group and 960 ± 30 mm Hg/s in the MT_{SS}

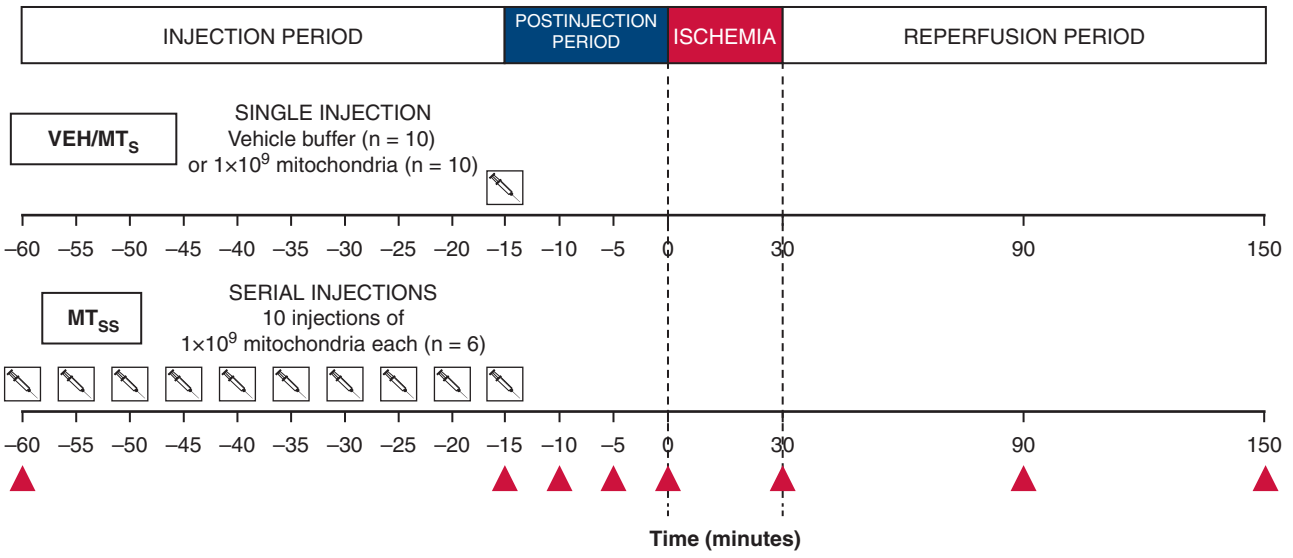


FIGURE 2. Experimental groups. The animals were divided into 3 groups: animals receiving vehicle solution alone (*Veh*, 6 mL), animals receiving single injections of mitochondria into the left main coronary artery (MT_S, 1×10^9 in 6 mL; n = 10), and animals receiving serial injections of mitochondria (MT_{SS}, 10 injections of 1×10^9 in 6 mL for each injection; n = 6) delivered every 5 minutes. Animals were allowed to recover for 15 minutes after the last injection. Temporary regional ischemia and reperfusion are as described in Figure 1. Injections in the MT_S and MT_{SS} groups are illustrated with syringe figures. Regional and global measurement timepoints are annotated (*red arrowheads*).

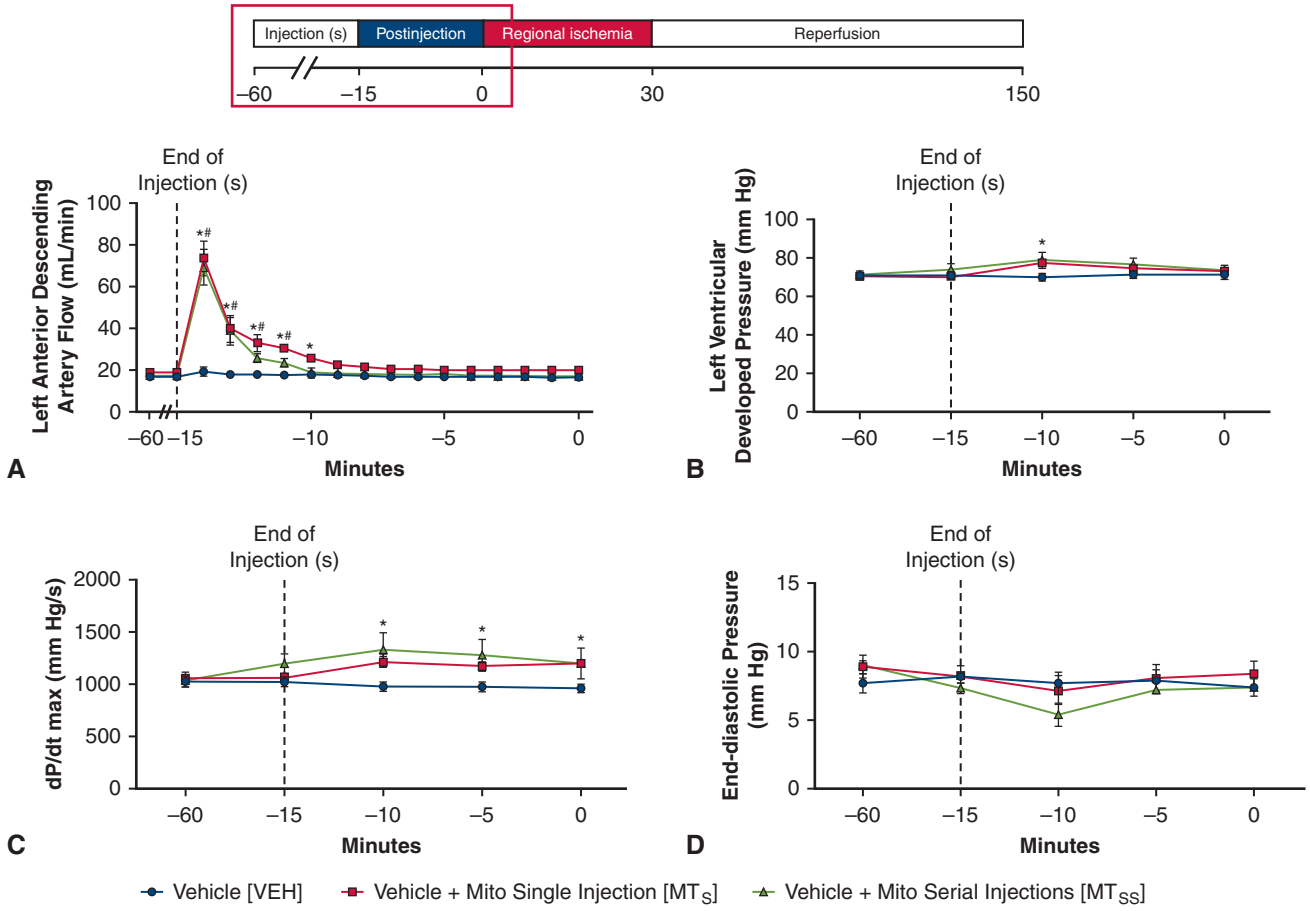


FIGURE 3. Global function after intracoronary injection of vehicle or vehicle containing mitochondria. A, Left anterior descending artery flow. B, Left ventricular developed pressure. C, Maximal rate of rise of left ventricular pressure. D, End-diastolic left ventricular pressure. Results are shown for the *red boxed area* only. Injections are denoted by *dashed vertical lines*. All data are mean ± SE for each group. **P* < .05, single injection vs vehicle; #*P* < .05, serial injections vs vehicle. dP/dt_{max}, Maximal change in pressure over time.

group, compared with 771 ± 34 mm Hg/s in the Veh group ($P = .02$ and $P = .004$ vs Veh, respectively) (Figure 4, *C*).

After 120 minutes of reperfusion, LV Ped was significantly decreased in the MT_S and MT_{SS} groups (8.0 ± 0.6 mm Hg and 8.2 ± 0.1 mm Hg, respectively; $P < .04$ for each), compared with 11.8 ± 1.3 mm Hg in the Veh group (Figure 4, *D*). No significant differences in LVEF, LV Pdev, LV dP/dt max, or LV Ped were seen between the MT_S and MT_{SS} groups after 120 minutes of reperfusion (Figure 4).

Postischemia Comparison: Regional Function After MT and a Subsequent Ischemic Event

Regional echocardiographic analysis at 120 minutes after reperfusion showed significantly enhanced regional function (Table E2). Fractional shortening was increased in both the MT_S (26.7 ± 1.8%; $P < .001$ vs Veh) and MT_{SS} (25.0 ± 2.6%; $P = .04$ vs Veh) groups compared with the Veh group (17.0 ± 1.0%) (Figure 5, *B*).

The 2-dimensional global strain analysis at 120 minutes of reperfusion was -18.5 ± 0.8% in the MT_S group ($P = .002$ vs Veh) and -20.9 ± 1.1% in the MT_{SS} group ($P = .003$ vs Veh), compared with -12.9 ± 0.8% in the Veh group (Figure 5, *C*).

Segmental shortening by sonomicrometry following 120 minutes of reperfusion was significantly increased in both the MT_S and MT_{SS} groups (11.1 ± 1.2% and 11.8 ± 0.5%, respectively), compared with 7.9 ± 0.5% in the Veh group ($P = .03$ and $P < .001$ for MT_S and MT_{SS} respectively vs Veh) (Figure 5, *D*).

CBF

No differences in CBF were observed within or between groups during equilibrium and at the end of ischemia (Table E2). CBF was significantly increased throughout

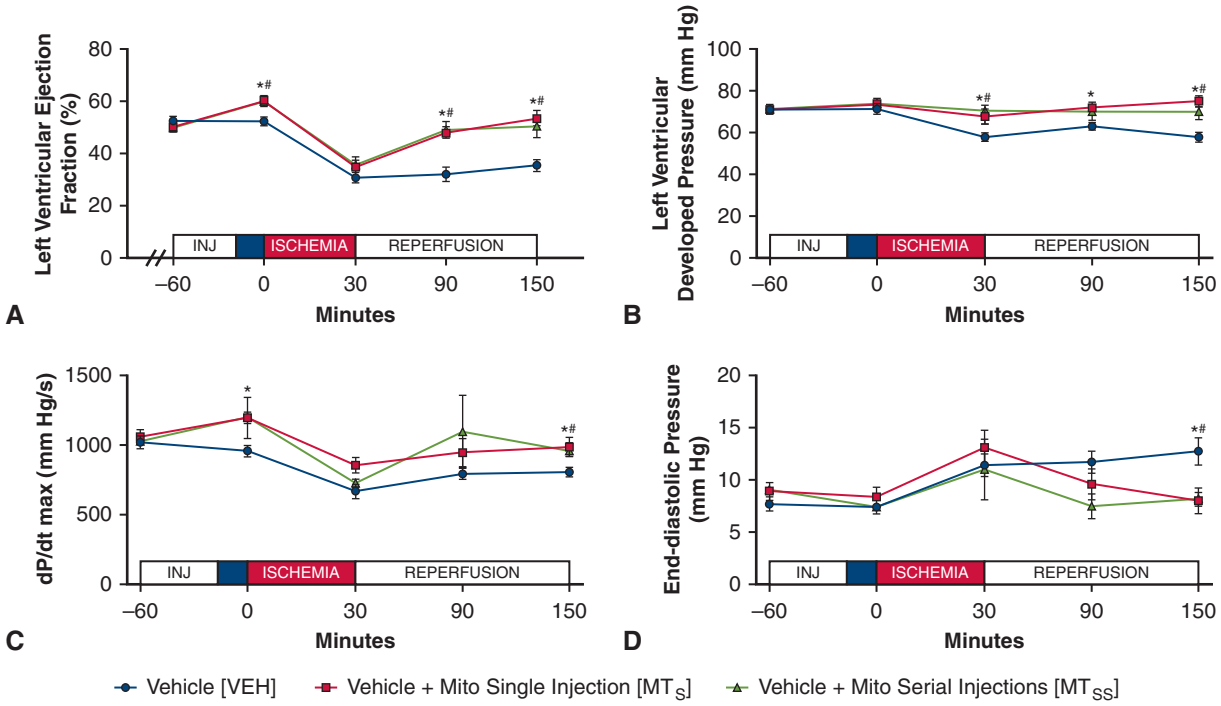


FIGURE 4. Global function during injection (INJ) and postinjection (*blue box*), regional ischemia (*red box*), and reperfusion (*white box*). A, Left ventricular ejection fraction. B, Left ventricular developed pressure. C, Maximal rate of rise of left ventricular pressure. D, End-diastolic left ventricular pressure. All results are mean ± SE for each group. *$P < .05$, single injection vs vehicle; #$P < .05$, serial injections vs vehicle. dP/dt_{max}, Maximal change in pressure over time.

reperfusion in both the MTs and MT_{SS} groups compared with the Veh group ($P = .04$ each at 120 minutes of reperfusion) (Figure 5, *A*). No differences in HR and MAP related to the increased CBF were observed within or between groups (Figure E2).

AAR/Infarct Size

The left ventricular AAR (% of LV mass) was 43.6 ± 2.1% in the MT_S group, 44.6 ± 2.8% in the MT_{SS} group, and 40.6 ± 1.5% in the Veh group (Figure 6, *A*). No significant difference in AAR was observed within or between groups. No significant difference in the wet weight-to-dry weight ratio was observed between groups (MT_S, 40.9 ± 0.1% vs Veh, 30.5 ± 0.2%, $P = .51$; MT_{SS}, 31.5 ± 0.1% vs Veh, $P = .8$) (Figure 6, *B*).

Infarct size (%AAR) was 37.9 ± 1.8% in the Veh group and was significantly decreased to 3.8 ± 0.5% in the MT_S group ($P < .001$ vs Veh) and to 4.2 ± 0.5% in the MT_{SS} group ($P < .001$ vs Veh) (Figure 6, *C* and *D*). There was no significant difference in %AAR between the MT_S and MT_{SS} groups ($P = .55$).

Histology and Transmission Electron Microscopy

Hematoxylin and eosin staining showed significantly less necrosis and edema in the MT groups compared with the Veh group (Figure E3). Electron microscopy confirmed mitochondrial damage and contraction bands in Veh hearts that were not present in MT hearts (Figure 7). In Veh hearts, mitochondria demonstrated a swollen shape, electron translucence, greater intermembrane space, enlarged ridges, and disrupted matrix with calcium accumulation (Figure 7, *A*). MT hearts showed preserved mitochondrial structure and only traces of calcium accumulation (Figure 7, *B* and *C*).

DISCUSSION

Investigations and the corresponding attempts at therapeutic interventions have consistently supported a prominent role of mitochondria in the response to IRI. This led us to the hypothesis that mitochondria may be a primary target for myocardial recovery and cardioprotection. Despite the advent of pharmacologic, genetic, and procedural therapies in preclinical studies, subsequent clinical studies have reported equivocal or negative results.[23] Rather than targeting a single mediator of the pathways leading to mitochondrial damage after IRI, transplantation of autologous mitochondria has been proposed and studied.[6-19]

In our initial studies, autologous mitochondria were injected directly into the ischemic zone of the myocardium at the time of reperfusion, showing significant improvement in infarct size and myocardial function.[6,7,9,14,18] Although

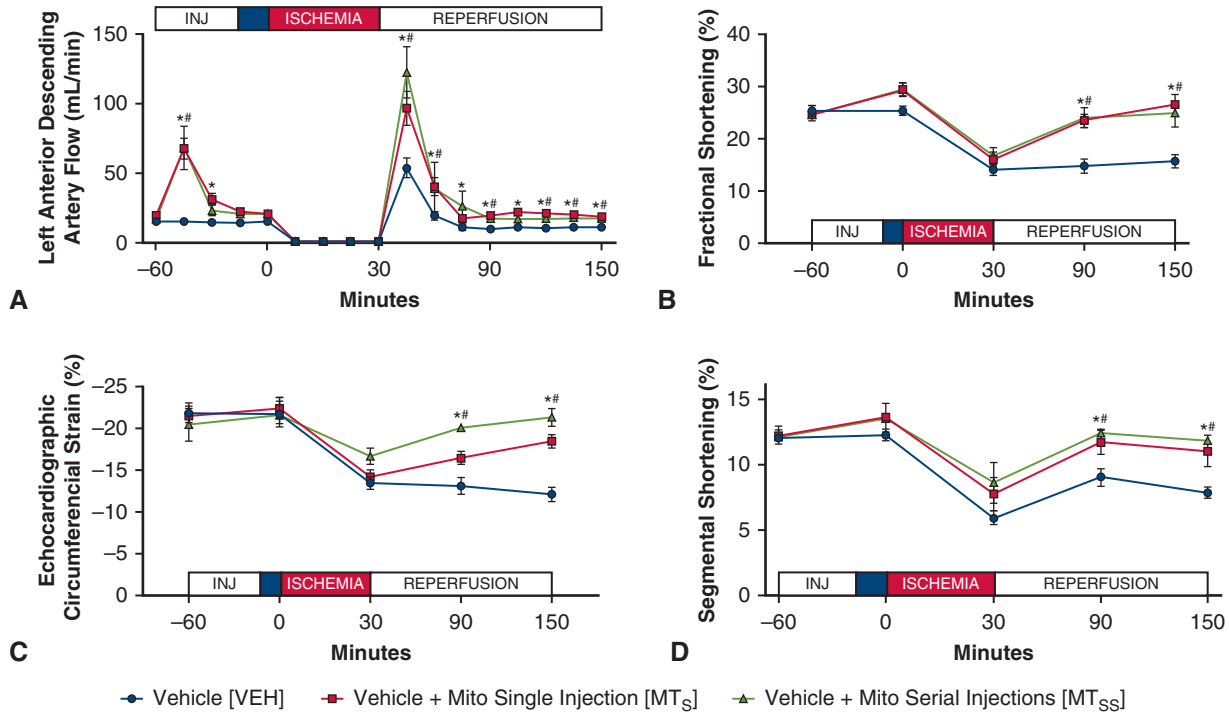


FIGURE 5. Regional function during injection (INJ) and postinjection (*blue box*), regional ischemia (*red box*), and reperfusion (*white box*). A, Left anterior descending artery flow. B, Echocardiographic fractional shortening. C, Echocardiographic left ventricular endocardial global circumferential strain. D, Left ventricular systolic segmental shortening. All results are mean ± SE for each group. $*P < .05$, single injection vs vehicle; $\#P < .05$, serial injections vs vehicle.

direct injection is practical for many applications, multiple injections are needed for global distribution, and direct access to the heart is required. For this reason, vascular delivery via intracoronary infusion has been investigated and validated.[10]

In the present study, we used 2 protocols: a single bolus intracoronary injection of mitochondria consisting of 1×10^9 mitochondria, delivered 15 minutes before the ischemic insult and serial intracoronary injections of 1×10^9 mitochondria/each every 5 minutes. These concentrations were based on previous studies demonstrating that 2×10^5, 2×10^6, and 2×10^7 mitochondria per gram wet weight provided equivalent cardioprotection.[7] To reconfirm these findings, we used 2×10^5 mitochondria per gram wet weight for single injection studies. In serial studies, this concentration was increased to 2×10^6 mitochondria per gram wet weight in total. Postischemic functional recovery indices were not significantly different with increased mitochondrial concentrations (serial injections). Both groups demonstrated reduced infarct size and restoration of function at the end of the reperfusion period (Figure 8). Vehicle alone has never previously demonstrated a protective benefit; therefore, serial injections of vehicle were not investigated. Although a single injection is a rapid process taking approximately 5 seconds, serial injections require 60 minutes. Given the time and the greater amount of tissue needed to perform serial injections, no benefits were identified using this approach.

In recent studies, we have shown that intracoronary delivery of mitochondria increases CBF in a concentration-dependent manner, with maximal increased CBF achieved with 2×10^5 mitochondria per gram wet weight or 1×10^9 mitochondria.[24] Mitochondrial concentrations of 2×10^6 and 2×10^7 mitochondria per gram wet weight did not increase CBF further beyond that achieved with 2×10^5 mitochondria per gram wet weight. The increase in CBF did not increase HR or MAP. In the present study, we observed a prolonged and repeatable increase in CBF with no increase in HR or MAP, in agreement with our previous results.[24]

The mechanisms by which MT provides myocardial protection have yet to be fully elucidated. This requires further investigation and was beyond the scope of this study. Previous investigations have shown that the transplantation of autologous mitochondria is associated with the induction of beneficial cytokines and proteomic expression, which further increases cardiomyocyte adenosine triphosphate content and up-regulates cardioprotective cytokines.[9,12]

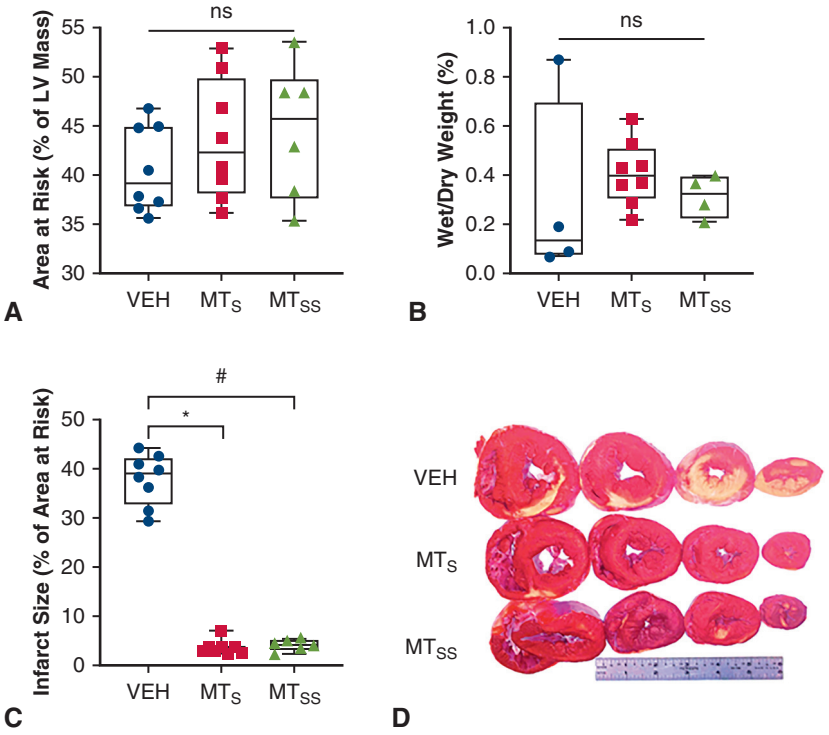


FIGURE 6. Area at risk (AAR) and infarct size. A, AAR as percentage of left ventricular mass. B, Wet weight–to–dry weight ratios. C, Infarct size as % of AAR. D, Representative examples of the infarct size determined by triphenyl tetrazolium chloride staining in the vehicle group (*Veh*; *top*), single mitochondria injection (MT_S; *middle*), and serial mitochondria injections (MT_{SS}; *bottom*). All results are mean ± SE for each group. *$P < .05$, MTS vs Veh; #$P < .05$, MTSS vs Veh. Ns, no significant difference at $P < .05$ detected. *LV*, Left ventricular

Several possible clinical scenarios were identified as possible applications of a preischemic MT: cardiac transplantation, cardiac procedures with expected prolonged cross-clamp times, procedures involving hearts with marginal function, interventional catheter-based procedures at high risk of ischemia, and procedures in which cardioplegic

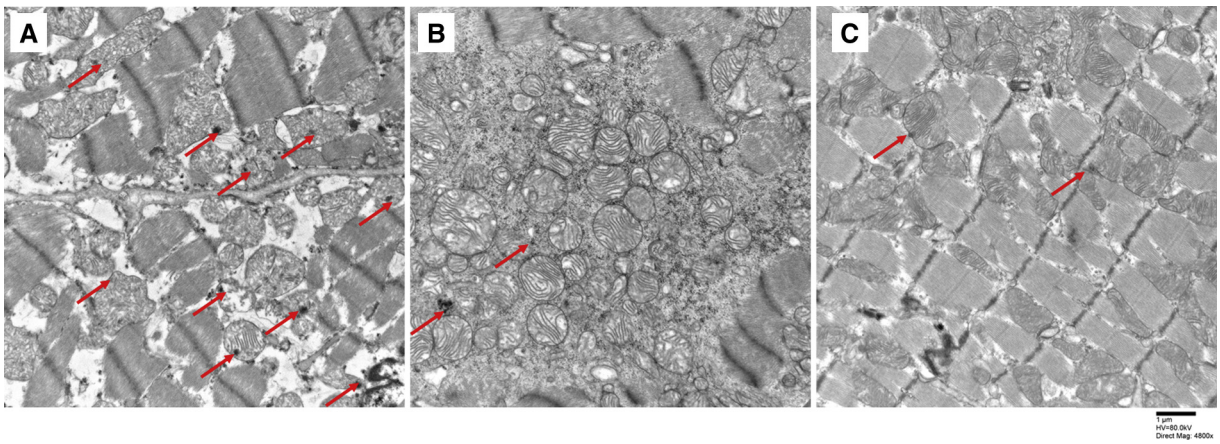


FIGURE 7. Representative electron microscopy images of the 3 experimental groups. A, Electron microscopy analysis showing contraction bands and electron translucent and swollen mitochondria, with a greater intermembrane space, enlarged ridges, disrupted matrix, and calcium accumulation (*arrows*) in vehicle-injected hearts, indicative of greater cell damage. Hearts treated with single mitochondria injection (B) and serial mitochondria injections (C) showed preserved mitochondrial structure with sparse calcium accumulation. Print magnification: 17,500× at 7.0 in. Hamamatsu ORCA HR camera; exposure, 3000 ms; gain, 1.7; bin, 1; gamma, 1.00; no sharpening; normal contrast.

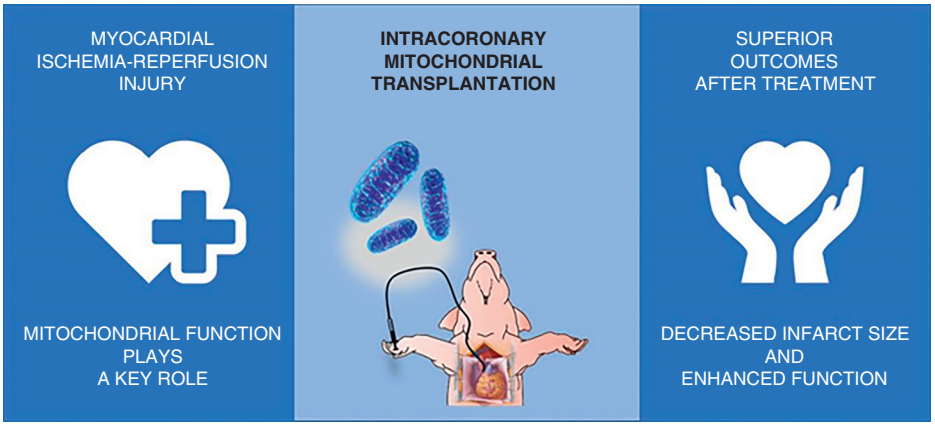


FIGURE 8. Mitochondrial transplantation protects against myocardial ischemia-reperfusion injury. Ischemia-reperfusion events severely alter mitochondrial structure and function, which play key roles in myocardial cell homeostasis. The transplantation of autologous mitochondria, isolated from the patient's own body and then injected intracoronary during the preischemic period, increases the function of the native mitochondria damaged during ischemia and improves the functional recovery of postischemic myocardium and cell viability during reperfusion.

protection is not ensured. Although this initial study suggests a potential benefit of MT, confirmatory prospective randomized clinical trials are needed to establish stronger evidence.

Study Limitations

Only female animals were used in our study, so as to reduce any possible effects related to urinary catheterization, which is less traumatic in females than males. The model included an RI and not a global IRI, and thus global compensatory recovery might have affected our results. We have previously demonstrated, using ^{18}F-R6G-labeled mitochondria and decay-corrected measurements, that 77.3 $\pm$ 5.5% of the transplanted mitochondria delivered by intracoronary injection remain contained within the injected hearts throughout reperfusion.[10]

In addition, we only reperfused the hearts for 2 hours after the end of ischemia; long-term efficacy studies are needed for verification. We also used young, otherwise healthy animals, thus eliminating confounding variables that could possibly be related to coexisting diseases. At present, the mechanism(s) modulating prophylactic MT remain to be fully elucidated in future investigations.

CONCLUSIONS

In conclusion, preischemic MT via intracoronary injection provides prophylactic myocardial protection from IRI, significantly decreasing myocardial infarct size and enhancing myocardial function (Figure 8). This novel technique is safe and has considerable potential to reduce morbidity and mortality in patients with a known risk of IRI. Although both single and serial delivery of mitochondria showed a benefit, serial injections did not provide significantly superior outcomes compared with single injection.

Webcast

You can watch a Webcast of this AATS meeting presentation by going to: https://aats.blob.core.windows.net/media/19%20AM/Sunday_May5/206BD/206BD/S40%20-%20Translational%20Research%20That%20will%20change/S40_6_webcast_081609766.mp4.

Conflict of Interest Statement

Dr Guariento reports patents issued (''The Method for Mitochondrial Isolation'' [WO 2015/192020A1] and ''The Use of Mitochondria for Clinical Purposes'' [PCT/US2015/035584]) and a connection to Cell Vitae Science with no compensation. Dr McCully reports a connection to Cell Vitae Science and patents issued (WO 2015/192020A1 and PCT/US2015/035584; Boston Children's Hospital). Dr del Nido is a cofounder of CellVitae Science and reports a patent pending (''Therapeutic Use of Mitochondria and Combined Mitochondrial Agent''). All other authors have nothing to disclose with regard to commercial support.

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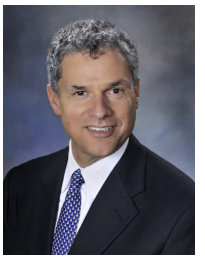
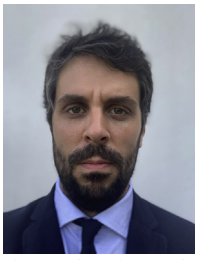
Key Words: ischemia/reperfusion injury heart, myocardial ischemia, myocardial protection

Discussion



Dr Todd K. Rosengart *(Houston, Tex)*. Thank you for that great work, and I appreciate you sharing the paper in advance. This is at least the second time I have had the privilege of reviewing this work by Dr del Nido's lab, and I find it fascinating. In prior publications and discussions, you posited that the mechanism of action in this mitochondrial transplantation is essentially a rescue technique; it is essentially taking healthy, viable mitochondria to replace those that are damaged by the ischemia or the ischemic event. This is different, though. You are pretreating with the mitochondria, so presumably they would be exposed to the same ischemia-reperfusion effects as the naive or the native mitochondria.

So why is this working?



Dr Alvise Guariento *(Boston, Mass)*. Thank you Dr Rosengart. This is really the key question. In terms of the mechanism, we still have no definitive answers, but we think that somehow the mitochondria we are injecting can change the balance in cell homeostasis. What we do know is that mitochondria can enhance the proteomic expression of some important cytokines. These data were obtained from what you just mentioned, postinjection studies. It is therefore possible that they act in the same way after a preischemia injection, somehow activating preconditioning pathways or other pathways that can induce an enhancement in cell function during the ischemic phase. We also noticed that during the ischemic phase, we could obtain better results when we injected them before. This was actually not the first study that we did with this strategy. In fact, we did a similar study where we injected mitochondria before a prolonged period of cold ischemia, and we had similar results. So I totally agree with you that understanding the mechanism should be the next step of our research.

Dr Rosengart. So one alternative possibility—and I apologize, I have not looked through all your articles to the extent to know whether or not you have looked at this—is perhaps the skeletal muscle mitochondria are different in some way than the cardiac. So, have you looked beyond the skeletal? Have you looked at, A, is there a difference and, B, are there other tissue that might be equally relevant?

Dr Guariento. In the early stages of developing this technology, we did a bunch of studies where we added mitochondria obtained from either skeletal muscle or liver, and also cardiac mitochondria. We didn't notice a great

difference in terms of ATP production or oxygen production. So, there may be some reasons for this, but we don't know yet.

Dr Rosengart. I will ask one last quick question before Dr Sellke. So, the other thing that was dramatic about this paper was the very significant decrease in myocardial infarction. You rarely see that much improvement in any intervention. Is there any specific reason why you think that was so?

Dr Guariento. This strategy seems very effective. This is the only answer I can give for now. We usually considered mitochondrial transplantation as a replacement of the native damaged mitochondria. In this study, everything was different in terms of what we speculated in the past.

Dr Frank W. Sellke *(Providence, RI).* Remind me, have you looked at the effects of the mitochondrial injection postischemia, because it is difficult to predict when somebody is going to have a myocardial infarction? Have you injected the mitochondria after the onset of the ischemic event?

Dr Guariento. This is the first study in which we injected them before. All our previous studies were focused on postischemia strategies, both at the immediate start of reperfusion or in a delayed fashion two hours after reperfusion.

Dr Sellke. The other question I had is that with all the stem cell studies, the benefit is not because of developing new myocytes, but more due to a trophic effect. I was wondering if that could have an effect as well, rather than increased energy utilization? Maybe there is some trophic effect from these transplanted mitochondria.

Dr Guariento. I totally agree with you. We know that in recent studies, others showed that stem cell infusion can also be closely related to some sort of mitochondrial mechanism. They didn't call this process mitochondrial transplantation but instead called it mitochondrial transfer, but the concept is quite the same.

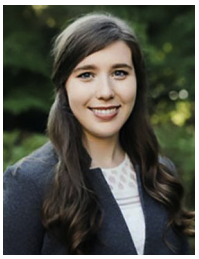
Dr Sellke. A very nice study.

Dr Guariento. Thank you very much, Dr Sellke.

Dr Marek A. Deja *(Katowice, Poland).* Maybe I missed it in the presentation, but what actually happened to these mitochondria? Do they survive in between the cells, do they get into the cells, how long do they survive? It is quite interesting, and I can't really understand, what are they doing there?

Dr Guariento. I didn't have the chance to show this. We investigated this in a previous paper, and the current study was mainly related to this new approach, the preischemia injections. We know that mitochondria can enter the cells within 5 minutes after the injection through an actin-dependent mechanism. We also know that they are rapidly integrated in the cells and they can fuse with the resident mitochondria. Subsequently, they can be found in the cells over a period of 28 days after injection. This is what we know so far.

We have also demonstrated this with F-18 rhodamine labeling of the mitochondria, and you can actually see them for quite a long period. Surely, a limitation of this study is that this is only a 2-hour reperfusion experiment, and we know that we will need to extend it to have more definitive results.



Katherine Driscoll *(Ithaca, NY).* I was thinking about this talk and then also the earlier talk on the increased mitochondrial DNA in pericardial fluid, and I was wondering if you could speculate on whether maybe the body has a mechanism kind of similarly mitochondrially related, and maybe that could be the related to why this therapy is working and also why you will see increased mitochondrial DNA in that area.

Dr Guariento. We know that we have tried to inject just components of the mitochondria, such as mitochondrial DNA, and have not achieved the same results.

APPENDIX E1. METHODS

Statistical Plan and Randomization

The number of experiments required for each group was determined by power analysis, with $\alpha = 0.05$ (2-sided) probability of type 1 error. Ignoring repeated measurements, 10 animals per group provided >95% power to detect a difference equal to twice the within- group SD ($\alpha = 0.05$, 2-sided) and 95% power to detect a difference equal to 2.5 times the within-group SD ($\alpha = 0.0095$, 2-sided, the Bonferroni-adjusted significance criterion for post hoc comparisons between groups). For the presence/absence of a finding, 10 animals per group would have had 95% power to detect a factor occurring in at least 26% of the animals. Based on preliminary studies, typically each experimental group consisted of 10 animals. A subsequent subgroup of 6 animal were used in the "mitochondria group" to test serial injections of mitochondria, assuming that for the presence or absence of a finding, 6 animals per group would have 95% power to detect a factor occurring in at least 40% of the animals.

Randomization based on a single sequence of random assignments was used in the study.

DISCUSSION

Mitochondrial Transplantation: History and Future Directions

The potential of autologous MT to reduce infarct size and enhancing myocardial function after IRI was initially assessed using both Langendorff-perfused and in situ blood-perfused rabbit hearts.[6,7,9] In these initial studies, autologous mitochondria were injected directly in 8 to 10 sites in the ischemic LV free wall. A cardioprotective effect from regional IRI was observed, with no adverse events, such as arrhythmia, changes in serial electrocardiography, or hypotension. Myocardial protection was also confirmed by decreased serum creatine kinase MB and cardiac troponin I levels compared with control hearts injected with respiration buffer alone.[14]

The internalization of the injected mitochondria was demonstrated in a variety of cardiac cells, including cardiomyocytes and fibroblasts. Further experiments with cell cultures showed that mitochondrial uptake occurs through actin-dependent endocytosis and results in the rescue of cellular function by increasing energy production and repairing mitochondrial DNA.[9] Transplanted mitochondria remain present and viable in the myocardium for 28 days.[7,14]

Uptake and distribution of the injected mitochondria was again confirmed by labeling isolated mitochondria with fluorescent proteins or gold nanoparticles, using 3-dimensional super-resolution microscopy and transmission electron microscopy. In a recently published study, we showed that isolated mitochondria are internalized in human cardiac cells within 5 minutes and then transported to early and late endosomes.[12] The majority of exogenous mitochondria escape these compartments and fuse with the endogenous mitochondrial network, whereas some organelles are degraded through hydrolysis.

Of great importance, transplantation of autogenic mitochondria does not induce any sign of autoimmunity, as recently demonstrated by our group. As a matter of fact, we found no direct, indirect, acute, or chronic alloreactivity to single or serial injections of syngeneic or allogeneic mitochondria.[19] In the same way, we also did not observe any damage-associated molecular pattern molecules to single or serial injections of syngeneic or allogeneic mitochondria.

Encouraged by our preliminary results with direct intramyocardial injection, we tested different methods of delivery, including intravascular coronary injection.[10] In preliminary studies, we demonstrated that vascular delivery is safe and provides for widespread distribution of the injected mitochondria throughout the heart. Electrocardiography and angiography showed no changes in coronary artery patency after injection. This method resulted in a significant decrease in myocardial infarct size and enhanced postischemic functional recovery, similar to the results obtained with direct injection of mitochondria.

Interest in this technique has been growing recently, with different studies demonstrating the potential of autologous MT in various diseases. For this reason, we recently started investigating the applications of this therapy in other organs subjected to IRI, including the kidneys, lungs, and skeletal muscle. We are also testing it for efficacy in different clinical scenarios, such as in diabetes mellitus. The injection of autologous mitochondria may provide for efficacious therapy to many of these organs, reducing morbidity and mortality. Although our initial studies suggest potential benefit of MT, confirmatory clinical trials are needed to verify our data.

Study Limitation: Mechanism of Action of MT

This study focused on the efficacy of preischemic MT against myocardial IRI. We investigated for the first time the delivery of mitochondria before ischemia, suggesting this as a viable option for protection against IRI. The evaluation of a potential mechanism of action was beyond the scope of this study and remains to be elucidated. However, we speculate that the isolated mitochondria being disconnected from the endoplasmic reticulum are not affected by nuclear–endoplasmic reticulum signaling and thus act independently of the ischemia–reperfusion signaling cascade.

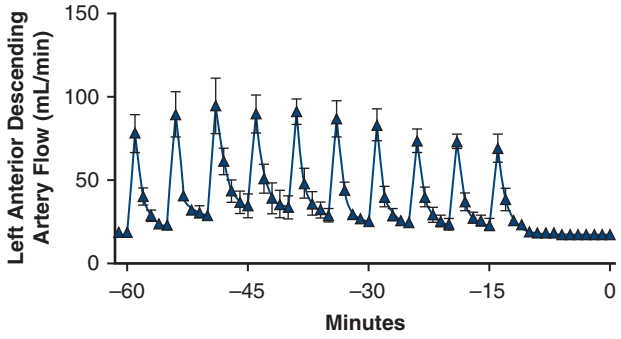


FIGURE E1. Left anterior descending flow during 10 serial injections of 1×10^9 mitochondria/each over 60 minutes. The increased coronary blood flow after mitochondrial transplantation remained consistent and reproducible in series, with an increased sustained after each injection. The dotted lines correspond to each mitochondrial injection.

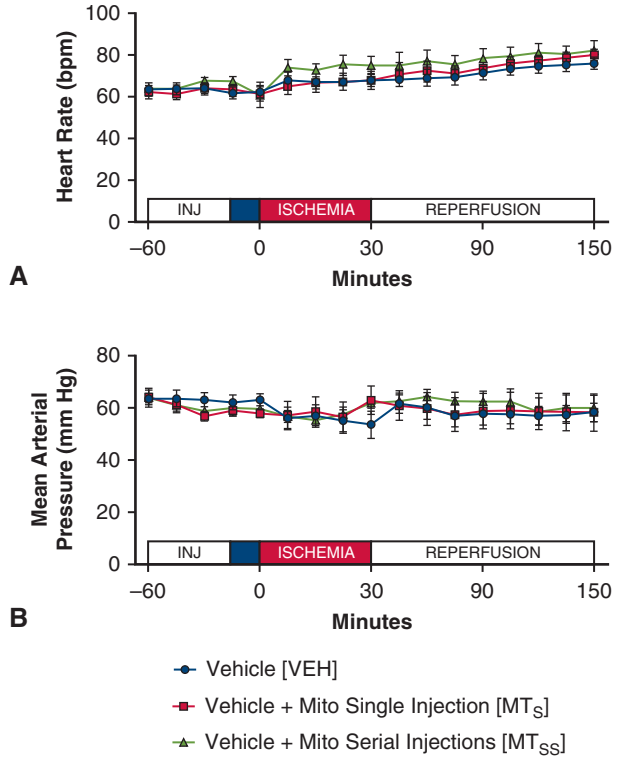


FIGURE E2. Heart rate (HR) and mean arterial pressure (MAP) in the 3 groups during the experiment. Single and serial mitochondria injections had no effect on HR (A) or MAP (B) compared with vehicle injection.

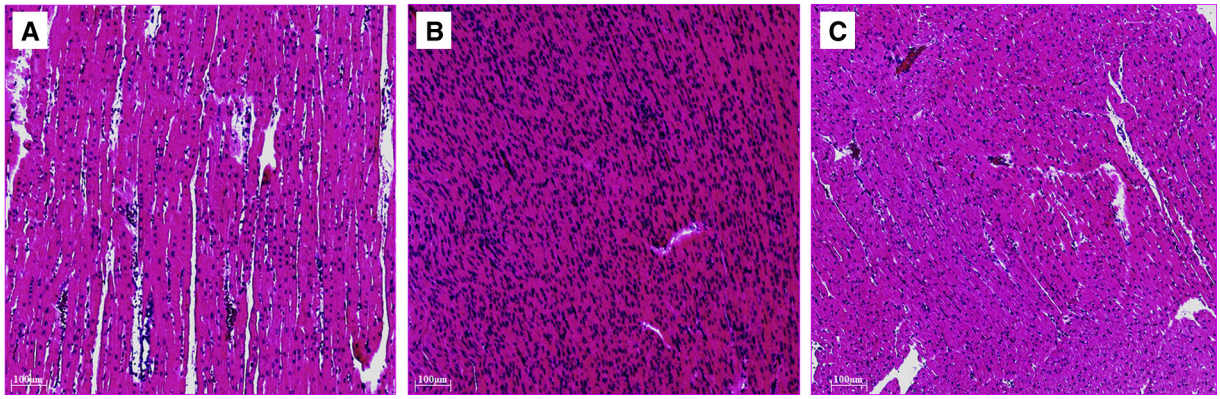


FIGURE E3. Myocardial tissue injury at the end of reperfusion. Representative hematoxylin and eosin–stained micrographs of heart graft tissue sections. Tissue sections from vehicle-injected hearts (A) show significantly more severe necrosis and edema compared with mitochondria-injected hearts (B and C).

TABLE E1. Measurements of global function during the entire experiment

Parameter	Group	Minutes				
		−60	**0**	**30**	**90**	**150**
LVEF, %	V	52.6 ± 1.6	52.3 ± 1.6	30.7 ± 2.0	32.1 ± 2.7	36.1 ± 2.1
	MT_S	50.2 ± 1.8	60.2 ± 2.1	34.8 ± 2.7	48.0 ± 2.4	53.6 ± 2.9
	MT_{SS}	49.9 ± 1.8	59.8 ± 2.1	35.9 ± 2.8	49.2 ± 3.0	50.3 ± 4.3
P value	MT_S vs V	.50	**.03**	.35	**.001**	**<.001**
	MT_{SS} vs V	.50	**.04**	.35	**.005**	**.04**
LVdevP, mm Hg	V	71.3 ± 1.6	71.1 ± 2.0	57.7 ± 2.0	63.2 ± 1.9	57.8 ± 2.4
	MT_S	70.4 ± 1.6	73.5 ± 1.7	67.7 ± 3.6	71.8 ± 2.2	74.9 ± 2.6
	MT_{SS}	71.6 ± 1.8	73.9 ± 2.4	70.5 ± 2.3	70.2 ± 4.3	69.9 ± 3.7
P value	MT_S vs V	.89	.61	**.04**	**.02**	**<.001**
	MT_{SS} vs V	.89	.61	**.004**	.27	**.03**
dP/dt max, mm Hg/s	V	1019 ± 58	961 ± 47	666 ± 60	796 ± 34	771 ± 34
	MT_S	1059 ± 52	1207 ± 50	855 ± 56	948 ± 99	988 ± 69
	MT_{SS}	1028 ± 31	1325 ± 165	726 ± 30	1095 ± 261	960 ± 30
P value	MT_S vs V	.90	**.003**	.10	.48	**.02**
	MT_{SS} vs V	.90	.26	.39	.48	**.004**
LVPed, mm Hg	V	7.7 ± 0.7	7.4 ± 0.9	11.4 ± 1.0	11.7 ± 1.0	11.8 ± 1.3
	MT_S	8.9 ± 0.9	8.4 ± 0.1	13.0 ± 1.7	9.6 ± 1.5	8.0 ± 0.6
	MT_{SS}	9.1 ± 0.3	7.4 ± 0.4	11.0 ± 2.9	7.5 ± 1.2	8.2 ± 0.1
P value	MT_S vs V	.44	.59	.91	.32	**.04**
	MT_{SS} vs V	.32	.98	.91	.23	**.04**

Statistically significant *P* values ($P < .05$) are shown in bold type. Minutes: -60, baseline; 0, end of preischemia; 30, end of ischemia; 90, first hour of reperfusion; 150, second hour of reperfusion. *LVEF*, Left ventricular ejection fraction; *V*, vehicle; MT_S, single injection; MT_{SS}, serial injections; *LVdevP*, left ventricular developed pressure; *dP/dt max*, maximum change in pressure over time; *LVPed*, left ventricular end diastolic pressure.

TABLE E2. Measurements of regional function during the entire experiment

Parameter	Group	Minutes				
		−60	0	30	90	150
LAD flow, mL/min	V	18.1 ± 1.6	17.3 ± 1.6	0.8 ± 0.3	10.2 ± 1.5	11.4 ± 1.8
	MT_S	19.2 ± 1.8	20.5 ± 1.5	1.0 ± 0.1	19.8 ± 1.6	19.0 ± 2.5
	MT_{SS}	17.9 ± 2.2	17.5 ± 0.2	1.6 ± 0.6	17.4 ± 1.7	17.8 ± 1.4
P value	MT_S vs V	.25	.26	.63	**.003**	**.04**
	MT_{SS} vs V	.52	.26	.25	**.01**	**.04**
LV echo FS, %	V	25.5 ± 0.9	25.4 ± 0.9	14.1 ± 1.0	14.8 ± 1.4	17.0 ± 1.0
	MT_S	24.7 ± 1.1	29.6 ± 1.3	16.1 ± 1.4	23.4 ± 1.3	26.7 ± 1.8
	MT_{SS}	24.5 ± 1.2	29.4 ± 1.4	16.7 ± 1.6	24.0 ± 1.9	25.0 ± 2.6
P value	MT_S vs V	.81	.05	.37	**.001**	**<.001**
	MT_{SS} vs V	.81	.08	.37	**.01**	**.04**
LV echo strain, %	V	-21.9 ± 1.1	-21.7 ± 1.1	-13.5 ± 0.7	-13.1 ± 0.9	-12.9 ± 0.8
	MT_S	-21.4 ± 1.3	-22.5 ± 1.3	-14.2 ± 0.9	-16.5 ± 0.7	-18.5 ± 0.8
	MT_{SS}	-21.5 ± 2.9	-22.4 ± 2.2	-16.2 ± 1.4	-20.0 ± 0.5	-20.9 ± 1.1
P value	MT_S vs V	.99	.10	.53	**.02**	**.002**
	MT_{SS} vs V	.99	.10	.53	**.001**	**.003**
LV segmental shortening, %	V	12.0 ± 0.3	12.3 ± 0.4	6.0 ± 0.5	9.0 ± 0.7	7.9 ± 0.5
	MT_S	12.3 ± 0.7	13.6 ± 1.1	7.8 ± 1.3	11.7 ± 0.9	11.1 ± 1.2
	MT_{SS}	12.1 ± 0.6	13.5 ± 1.2	8.7 ± 1.6	12.5 ± 0.3	11.8 ± 0.5
P value	MT_S vs V	.93	.61	.31	**.04**	**.03**
	MT_{SS} vs V	.93	.61	.31	**.002**	**<.001**

Statistically significant *P* values ($P < .05$) are shown in bold type. Minutes: -60, baseline; 0, end of preischemia; 30, end of ischemia; 90, first hour of reperfusion; 150, second hour of reperfusion. *LAD*, Left anterior descending artery; *V*, vehicle; MT_S, single injection; MT_{SS}, serial injections; *LV*, left ventricular; *FS*, fractional shortening.