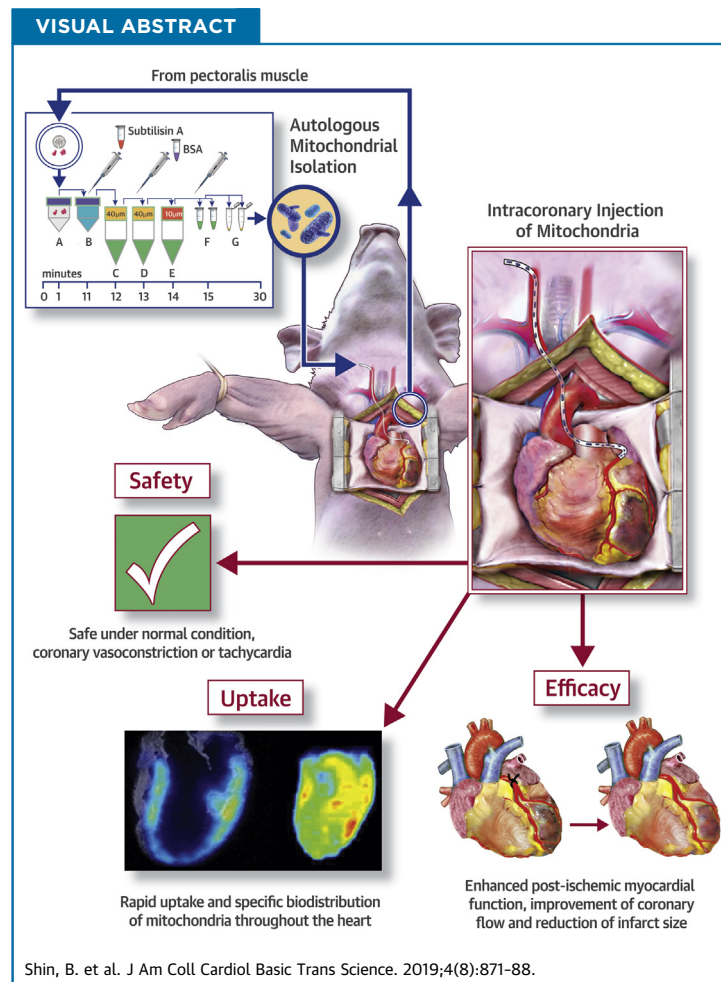


PRECLINICAL RESEARCH

A Novel Biological Strategy for Myocardial Protection by Intracoronary Delivery of Mitochondria: Safety and Efficacy



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ABBREVIATIONS AND ACRONYMS

ADH = antidiuretic hormone

ATP = adenosine triphosphate

AUC = area under the curve

CBF = coronary blood flow

K⁺ = potassium ion

K_{ATP} = ATP-sensitive
potassium channel

K_{IR} = Inwardly rectifying
potassium channel

LCA = left coronary artery

LV = left ventricular

HIGHLIGHTS

- Mitochondrial transplantation uses mitochondria isolated from the patient's own body to replace or augment native mitochondria damaged by ischemia reperfusion injury.
- The transplanted mitochondria can be delivered to the myocardium by intra-coronary injection.
- Intracoronary injection of mitochondria is safe and has no effect on coronary patency.
- Intracoronary injection of mitochondria provides for the rapid uptake and specific biodistribution of mitochondria throughout the heart.
- Intracoronary mitochondrial transplantation is efficacious and provides for enhanced post-ischemic myocardial function, improved coronary blood flow and reduction of infarct size.

SUMMARY

Mitochondrial dysfunction is the determinant insult of ischemia-reperfusion injury. Autologous mitochondrial transplantation involves supplying one's healthy mitochondria to the ischemic region harboring damaged mitochondria. The authors used in vivo swine to show that mitochondrial transplantation in the heart by intracoronary delivery is safe, with specific distribution to the heart, and results in significant increase in coronary blood flow, which requires intact mitochondrial viability, adenosine triphosphate production, and, in part, the activation of vascular K_{IR} channels. Intracoronary mitochondrial delivery after temporary regional ischemia significantly improved myocardial function, perfusion, and infarct size. The authors concluded that intracoronary delivery of mitochondria is safe and efficacious therapy for myocardial ischemia-reperfusion injury. (J Am Coll Cardiol Basic Trans Science 2019;4:871-88) © 2019 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Ischemic heart disease is one of the leading causes of global morbidity and mortality (1). Mitochondrial damage is the principal pathogenesis of myocardial ischemia-reperfusion injury leading to cardiomyocyte death and contractile failure (2-7).

We have previously developed a novel therapy, autologous mitochondrial transplantation, in which viable, respiration-competent mitochondria from nonischemic tissue from the patient's own body are isolated and then transplanted into the ischemic myocardium to ameliorate the effects of mitochondrial damage experienced by the ischemic region. The therapeutic efficacy of mitochondrial transplantation was demonstrated in a series of animal studies (8-14) and showed promise in a recent human application

(15). In both animals and humans, post-ischemic transplantation of healthy mitochondria by direct injection into the myocardium results in significant improvements of contractile function and tissue viability of the injured myocardium.

The transplanted mitochondria are readily internalized by cardiac cells (11) by actin-dependent endocytosis (12), with rapid cytosolic transition and fusion with the endogenous mitochondrial network (13). The transplanted mitochondria act to increase myocardial adenosine triphosphate (ATP) levels, upregulate proteomic pathways for mitochondrial function, upregulate myoprotective cytokines, and replace damaged mitochondrial deoxyribonucleic acid (8-10).

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The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the JACC: Basic to Translational Science [author instructions page](#).

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Although mitochondrial transplantation by direct tissue injection of donor mitochondria is therapeutically effective, it harbors limitations in that multiple injections and physical manipulations of the heart are necessary for adequate distribution of mitochondria throughout the heart. Open access to the heart is also required, which significantly limits the potential patient population who could undergo mitochondrial transplantation.

Minimally invasive, intracoronary delivery of mitochondria can avoid these drawbacks and disseminate mitochondria to the entire region that was infused, allowing an entry for whole-organ mitochondrial therapy. Previous studies using an isolated perfused heart model have demonstrated that intracoronary delivery of mitochondria results in rapid uptake and global distribution of the transplanted mitochondria (11). However, preclinical in vivo evaluation of the biodistribution, safety, and efficacy of intracoronary delivery of mitochondria is imperative for clinical translation and the expansion of therapeutic applications.

In the present report, we investigated the biodistribution and the safety of a catheter-based, intracoronary mitochondrial transplantation and evaluated the therapeutic efficacy in treating regional myocardial ischemia-reperfusion injury in the clinically relevant in vivo swine model.

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METHODS

All procedures conformed to institutional guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of Boston Children's Hospital.

EXPERIMENTAL DESIGN. A total of 57 adult female Yorkshire swine (45.0 ± 5.5 kg) were used. This study was conducted in 3 phases. In the first phase, the safety profile and biodistribution of mitochondria by intracoronary delivery was determined in the non-ischemic swine. In the second phase, based on the first-phase finding of the increase in coronary blood flow (CBF) from intracoronary infusion of mitochondria, the mechanism of mitochondria-induced increase in CBF was investigated. Finally, in the third phase, the efficacy of intracoronary mitochondrial transplantation in providing cardioprotection after regional myocardial ischemia was evaluated.

SURGICAL PREPARATION AND MITOCHONDRIAL ISOLATION. Animals were sedated with Telazol (Zoetis, Parsippany, New Jersey) (2.2–4.4 mg/kg)/xylazine (Anased, Greeley, Colorado) (1–2 mg/kg) and intubated. General anesthesia was maintained with a

0.5% to 2% isoflurane-oxygen mixture. Ventilation was adjusted to maintain pH 7.35 to 7.45, P_{CO_2} 30 to 40 mm Hg, and P_{O_2} 85 to 100 mm Hg. Core temperatures were maintained at $>36^\circ\text{C}$. Median sternotomy was performed, and the heart was suspended in a pericardial cradle. Then, angiographic access to the left coronary artery (LCA) was established by floating a 5-F JR angiography catheter (Merit Medical Systems, Inc., South Jordan, Utah) through the right carotid artery (5-F sheath) to the left coronary ostium under fluoroscopy. The coronary tree was visualized by injection of 5 ml of contrast solution (74% Ioversol Optiray-350, Mallinckrodt, Inc., St. Louis, Missouri) during 5 s, followed by a 5-ml saline flush. Two pieces of muscle were harvested from the pectoralis major muscle of each animal with a 6-mm biopsy punch and immediately used for mitochondrial isolation. Autologous mitochondria were isolated and mitochondrial ATP content was measured as previously described (8,9,16).

PHASE 1: SAFETY OF INTRACORONARY DELIVERY OF MITOCHONDRIA AND BIODISTRIBUTION IN THE NONISCHEMIC HEART. A total of 20 animals were used in phase 1. There were no animal losses in phase 1 studies. Myocardial uptake and biodistribution of mitochondria by intracoronary delivery were evaluated in 3 animals. Next, 6 animals were used to evaluate the concentration tolerance of intracoronary injection of mitochondria by angiographic injection of 5 mitochondrial concentrations into the LCA. Intracoronary injection of 1 optimum mitochondrial concentration was then tested under normal conditions and in the presence of myocardial stressors, including coronary vasoconstriction, tachycardia, and increased afterload ($n = 6$). The safety of repeated injections of mitochondria was evaluated ($n = 5$). Coronary patency, CBF, hemodynamics, and regional and global left ventricular (LV) functions were evaluated.

Mitochondrial biodistribution and cellular uptake. To evaluate myocardial uptake and biodistribution of mitochondria, autologous mitochondria were labeled with ^{18}F -rhodamine-6G (11,17). To increase detection sensitivity, mitochondria were delivered at a concentration 6-fold greater (6×10^9) than the therapeutic dosage used in our previous studies (1×10^9). Mitochondria were injected serially in 6 5-s boluses, each bolus containing 1×10^9 mitochondria in 5 ml of vehicle (300 mM sucrose, 10 mM K^+ 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid pH 7.2, and 1 mM K^+ ethylene glycol-bis(β -aminoethyl ether)- N,N,N' -tetraacetic acid, pH 8.0; Sigma Aldrich, St. Louis, Missouri). After 10 min of circulation, a timeframe long enough for the blood to circulate through the entire body approximately 10 times (18), the animal

was euthanized and imaged by whole-body positron emission tomography (Siemens, Munich, Germany) ($n = 2$). In a separate animal, cellular uptake of mitochondria was evaluated by intracoronary injection of xenogeneic mitochondria isolated from human cardiac fibroblasts and labeled with iron (II, III) oxide nanoparticles. The use of human mitochondria allows differentiation of the transplanted mitochondria from the native swine cardiac mitochondria by immunohistochemistry. Transplanted mitochondria were detected by Prussian blue staining for iron-labeled mitochondria and immunohistochemistry as previously described (9,11) ($n = 1$).

Safety of intracoronary injection of mitochondria. Five concentrations of mitochondria (1×10^3 , 1×10^5 , 1×10^7 , 1×10^9 , and 1×10^{11}) were each suspended in 5 ml of vehicle. Each concentration was injected into the LCA as a 5-s bolus followed by a 5-ml saline flush in the order of increasing concentration ($n = 6$).

To evaluate the safety of intracoronary injection of mitochondria in the presence of increased myocardial demand, coronary vasoconstriction or tachycardia with increased afterload was individually induced in 6 animals. Coronary vasoconstriction was induced by intracoronary injection of antidiuretic hormone (ADH) (Par Pharmaceutical, Woodcliff Lake, New Jersey; and Sigma Aldrich) (1.75 nmol in 5 ml of saline). Tachycardia with increased afterload was induced with epinephrine ($0.5 \text{ } \mu\text{mol}$ in 5 ml of saline) (Patterson Veterinary, Devens, Massachusetts; and Sigma Aldrich). On confirmation of the intended effects, 1×10^9 mitochondria were provided as boluses into the LCA ($n = 6$). Then, in 5 animals, the safety of repeated injection of mitochondria was assessed by 10 serial intracoronary injections of 1×10^9 mitochondria in 5 ml of vehicle into the LCA in 5-s boluses every 5 min.

Assessment of coronary patency, coronary blood flow, and cardiac function. Coronary patency was evaluated by angiography immediately after and 5 min after intracoronary mitochondrial injections. Angiographic analysis was performed by the Cardiology Department of Boston Children's Hospital. CBF was continuously measured by placing an ultrasonic flow probe (3R1334, Transonic Systems Inc, Ithaca, New York) circumferentially around a 5-mm to 7-mm segment of the left anterior descending artery distal to the first diagonal branch and recorded via a Transonic T206 blood-flowmeter. Global LV function was evaluated by a 7F-VSL transonic pressure-volume conductance catheter inserted into the LV cavity through the LV apex. Measurements were analyzed with LabChart 7 acquisition software (AD Instruments, Sydney, Australia). Regional myocardial function was evaluated

by sonomicrometry of the LV free wall and analyzed with SonoView post-processing software SonoLabD53 (Sonometrics Corp., London, United Kingdom) (19).

PHASE 2: EVALUATION OF MITOCHONDRIA-INDUCED INCREASE IN CORONARY BLOOD FLOW. A total of 21 animals were used in phase 2. There were no animal losses in phase 2 studies. We first evaluated whether the increase in CBF resulting from intracoronary injection of mitochondria was the result of increased myocardial oxygen consumption by the introduction of large amounts of mitochondria into the vasculature. This was done by 2 methods: first, by measuring the CBF in response to direct myocardial injection of mitochondria at 10 locations (1×10^8 each in 0.1 mL of vehicle) in close proximity to the left anterior descending artery, using a tuberculin syringe ($n = 3$); and second, by the measurement of coronary sinus proportion venous oxygen saturation at baseline, immediately after (at peak of increase in CBF) and 10 min after intracoronary injection of mitochondria ($n = 4$).

Intracoronary injection of devitalized mitochondria, HeLa and HeLa p^0 cell mitochondria. To investigate the role of mitochondrial viability and respiration competence in mitochondria-induced increase in CBF, mitochondria were devitalized (9) and injected into the LCA ($n = 4$). Next, mitochondria (1×10^9) isolated from HeLa- and HeLa- p^0 cells, which differ in their ability to perform oxidative phosphorylation (12), were injected separately into the left anterior descending artery ($n = 6$). CBF was compared. HeLa cells (CRM-CCL-2, American Type Culture Collection, Manassas, Virginia) and HeLa- p^0 cells were cultured as previously described (9,12).

Inhibition of coronary vasodilatory pathways. To determine the biochemical pathway(s) involved in mitochondria-induced increase in CBF, potential key mediators of coronary vasodilation were investigated in vivo, including endothelium-mediated nitric oxide synthase (20), cyclooxygenase (20) and vascular smooth muscle (SM) mediated adenosine receptors (21), ATP-sensitive potassium (K_{ATP}) channels (22), and inwardly rectifying potassium (K_{IR}) channels (23). CBF in response to intracoronary injection of 1×10^9 mitochondria was compared in the presence and absence of each pathway inhibition. Activators of each pathway were used as positive controls.

Animals were pre-treated separately by slow intracoronary infusion of increasing concentrations of the pedigreed blocker of each vasodilatory pathway ($n = 4$): nitromonomethyl L-arginine (0-100 μM ; nitric oxide synthase blocker) (Sigma Aldrich) (20), indomethacin (0-100 mM; cyclooxygenase blocker) (Sigma Aldrich) (20), 8-*p*-sulfophenyl

theophylline (0–1 mM; adenosine receptor blocker) (Sigma Aldrich) (21), glibenclamide (0–2 mM; K_{ATP} channel blocker) (Sigma Aldrich) (22), and barium chloride (0 to 100 μ M; K_{IR} channel blocker) (23). Nitromonomethyl L-arginine, 8-*p*-sulphophenyl theophylline, and barium chloride (Sigma Aldrich) were dissolved separately in 60 ml of saline. Stock solutions of glibenclamide and indomethacin were made in 1 ml of dimethyl sulfoxide (Sigma Aldrich) and slowly dissolved in 60 ml of warmed saline. A blocker was infused into the LCA during 20 min. Five min after completion of blocker treatment, pathway inhibition was confirmed by intracoronary injection of a known activator of the tested pathway. Bradykinin (0.01 nmol) (Sigma Aldrich) was used as cyclooxygenase and nitromonomethyl L-arginine pathway activator (24), nicorandil (50 μ mol) (Sigma Aldrich) was used as K_{ATP} -channel activator (25), and ATP (30 μ M) was used as K_{IR} -channel pathway activator (23). After confirmation of inhibition of the pathway in question, 1×10^9 mitochondria were injected into the LCA and CBF was measured. All blockers and activators were purchased from Sigma-Aldrich, St. Louis, Missouri).

PHASE 3: EFFICACY OF INTRACORONARY DELIVERY OF MITOCHONDRIA IN REGIONAL ISCHEMIA-REPERFUSION INJURY. A total of 16 animals were used in phase 3. There was 1 animal loss in the vehicle group. Animals were subjected to 30 min of regional myocardial ischemia by temporary snaring of the mid left anterior descending artery just distal to the second diagonal branch. The snare was released, and immediately on reperfusion, animals received either an intracoronary bolus of 1×10^9 mitochondria in 5 ml of vehicle ($n = 8$) or 5 ml of vehicle alone ($n = 8$). Hemodynamics, regional and global LV function, and CBF were continuously acquired. After 120 min of reperfusion, animals were euthanized, and the hearts were analyzed for area at risk and infarct size.

ECHOCARDIOGRAPHY. Echocardiography was acquired with a Philips iE33 machine (Philips Medical Systems, Andover, Massachusetts) with an X7-2 (7–2 MHz) transducer at pre-ischemia, at 30 min of ischemia, and after 1 and 2 h of reperfusion. Short-axis view and M-mode tracings at the midpapillary level were analyzed with a RadiAnt DICOM Viewer 5.02 (Medixant, Poznan, Poland) according to the American Society of Echocardiography standards (26).

EUTHANASIA. Animals were euthanized by intravenous injection of Fatal-Plus (50 μ g/kg).

AREA AT RISK AND INFARCT SIZE. Area at risk and infarct size were determined with tetrazolium chloride staining and planimetry analysis (7–9).

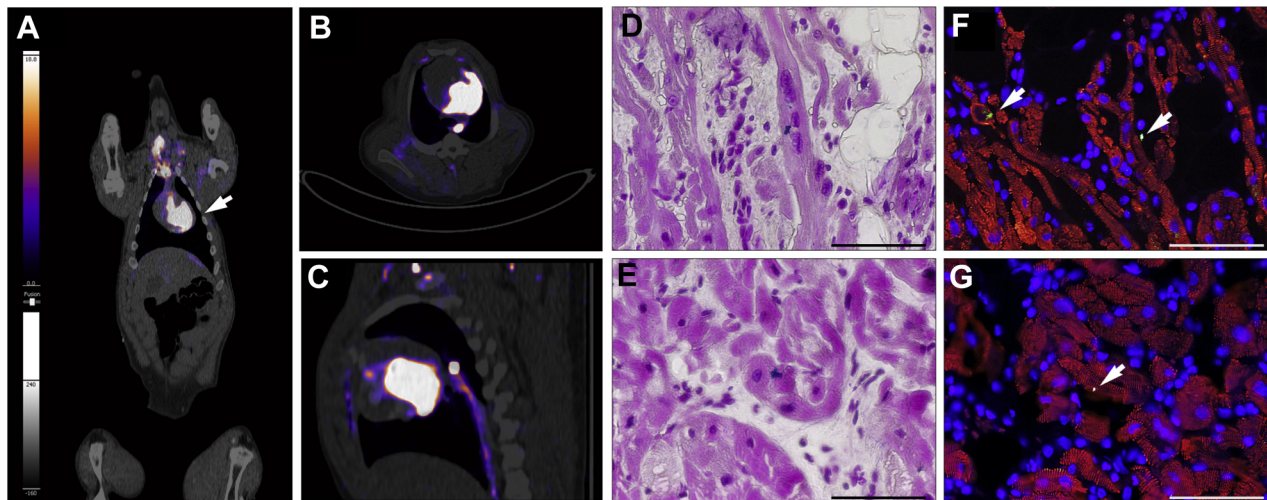
STATISTICAL ANALYSIS. Statistical analyses were performed with Stata software version 11.0 (Stata-Corp, College Station, Texas). All data are expressed as mean $\pm$ SE of the mean. Blinding was not adopted for data collection and analysis of different injectates. Continuous data (CBF, hemodynamics, and regional and global LV contractility) were compared between groups with 2-way repeated-measures analysis of variance. When the overall difference across groups was significantly different, a Bonferroni-adjusted post hoc analysis was used for pairwise comparisons of interest. For CBF, comparisons were made between each point and baseline CBF at time zero within each group. Area under the curve (AUC) was compared between groups with a 1-way analysis of variance, and results are presented as mean $\pm$ SE of the mean with 95% confidence interval. A 1-way analysis of variance was also used for echocardiographic analyses, area at risk, and infarct size. Statistical significance was claimed at a 2-sided $p < 0.05$.

RESULTS

PHASE 1: SAFETY AND BIODISTRIBUTION OF INTRACORONARY DELIVERY OF MITOCHONDRIA. Myocardial uptake and biodistribution of mitochondria. Myocardial uptake and biodistribution of mitochondria were evaluated by intracoronary injection of 18 F-rhodamine-6G-labeled mitochondria (6×10^9) in the LCA ($n = 2$). Whole-body positron emission tomographic scan images showed that the transplanted mitochondria were located specifically in the left ventricle (Figures 1A to 1C). 18 F-tracer signals were also present in the arterial sheath and in the right carotid artery where the coronary catheter was placed, and a small amount of tracer was detected in the descending aorta (Figures 1A to 1C). There was no evidence of significant tracer accumulation in any other organs.

Myocardial cellular uptake of mitochondria was demonstrated in a separate animal by intracoronary injection of iron (II, III) oxide-labeled human cardiac fibroblast mitochondria into the swine LCA ($n = 1$). Serial section immunohistochemistry and Prussian blue costaining of the swine heart confirmed the presence of human mitochondria in the heart tissue within cardiomyocytes, interstitial spaces, and the vascular walls (Figures 1D to 1G).

Intracoronary injection of mitochondria and myocardial function. There were no statistical differences between intracoronary injection of vehicle and baseline values for any of the hemodynamic parameters. Intracoronary injection of mitochondria did not affect heart rate, mean arterial pressure, or

FIGURE 1 Biodistribution and Myocardial Uptake of Autologous Mitochondria by Intracoronary Delivery

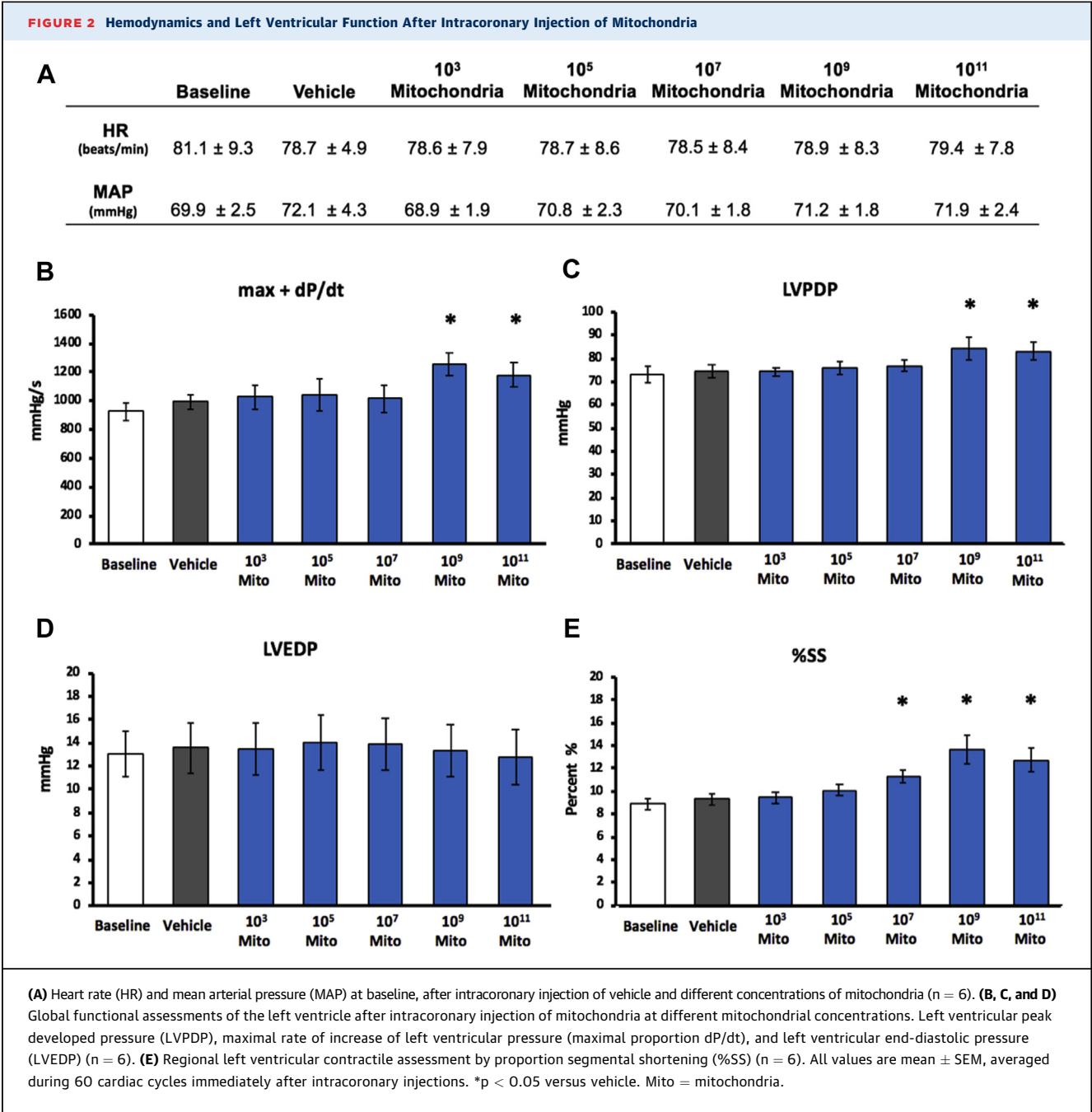
(A, B, and C) Representative positron emission tomography (PET) images 10 min after intracoronary injection of ^{18}F -rhodamine-6G iron (II, III) oxide nanoparticle-labeled mitochondria. Tracer accumulation is observed in the left ventricle (arrow) and along the coronary angiography catheter present through the right carotid arterial access (arrow). (D and E) Prussian blue stain of iron oxide-labeled human mitochondria transplanted into a swine myocardium. (F and G) Fluorescence immunohistochemistry of the transplanted mitochondria in consecutive slices of (D) and (E) (arrows). Green: antihuman mitochondria (MTCO2); red: antisarcomeric α -actinin; blue (4',6-diamidino-2-phenylindol [DAPI]): nuclei. Scale bars = 100 μm .

cardiac rhythm at any concentration of mitochondria tested compared with baseline or vehicle (Figure 2A). Higher concentrations of mitochondria (1×10^9 and 1×10^{11}) significantly enhanced regional and global LV function, as observed by increases in the proportion of LV free-wall segmental shortening (1×10^9 p = 0.016 vs. baseline and p = 0.015 vs. vehicle; 1×10^{11} p = 0.013 vs. baseline and p = 0.014 vs. vehicle) and +max dP/dt (1×10^9 p = 0.026 vs. baseline and p = 0.024 vs. vehicle; 1×10^{11} p < 0.001 vs. baseline and p < 0.001 vs. vehicle), and by peak LV developed pressure (1×10^9 p = 0.034 vs. baseline and p = 0.029 vs. vehicle; 1×10^{11} p = 0.035 vs. baseline and p = 0.028 vs. vehicle) (Figures 2B to 2E).

Intracoronary injection of mitochondria and CBF. Angiographic analyses showed patent coronary arteries with no detectable lesions or blockages (Figures 3A and 3B, Videos 1 and 2). Mean CBF before mitochondrial injection was 20.5 ± 1.3 ml/min (Figure 3C). Intracoronary injection of mitochondria increased CBF in a concentration-dependent manner (Figures 3C and 3D). Analysis of the AUC for CBF showed significant increases in CBF at mitochondrial concentrations of 1×10^7 ($10,001.7 \pm 914.3$ ml/min $\times$ s; p = 0.032), 1×10^9 ($19,843.7 \pm 1,208.4$ ml/min $\times$ s; p < 0.001), and 1×10^{11} ($18,262.3 \pm 2,131.6$ ml/min $\times$ s; p < 0.001) compared with vehicle alone ($7,242.9 \pm 624.7$ ml/min $\times$ s) (Figure 3D). Maximum CBF was

observed at a mitochondrial concentration of 1×10^9 , a 325% increase compared with baseline CBF (p < 0.001). Increase in CBF was sustained for 6.5 ± 0.6 min. No differences were observed in peak CBF (p = 0.842) or in the duration of increase (p = 0.304) between 1×10^9 and 1×10^{11} mitochondria (Figures 3C and 3D). The response of CBF was reproducible with repeated injections of mitochondria (1×10^9), every 5 min for 10 repetitions (Figure 3E). There were no effects on heart rate or mean arterial pressure with either single or serial injections of mitochondria (Figure 3F).

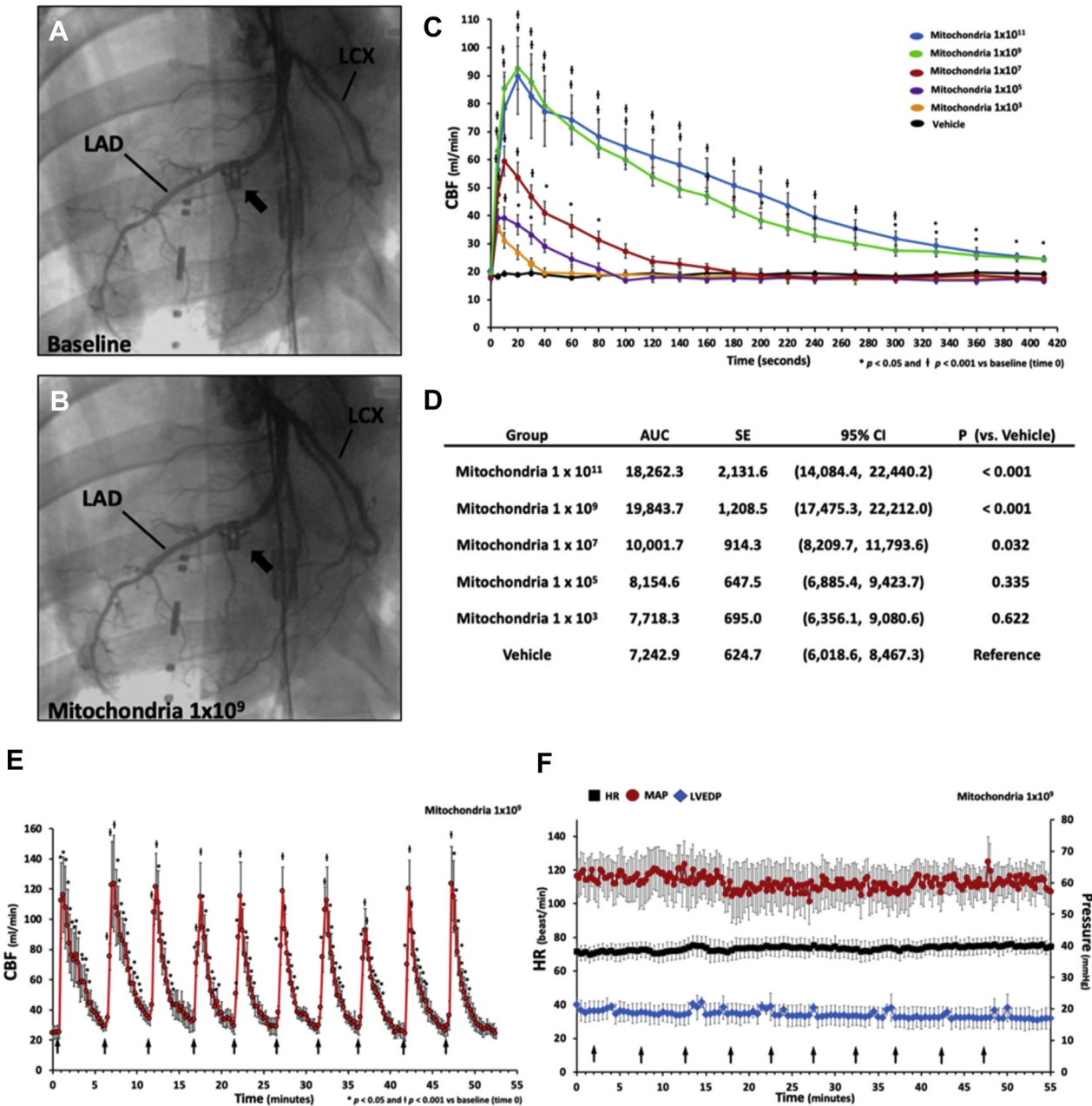
Intracoronary injection of mitochondria during increased myocardial demand. Intracoronary injection of ADH resulted in coronary vasoconstriction, leading to a significant decrease in CBF (p < 0.001) (Figures 4A and 4B). Intracoronary injection of mitochondria in the ADH-treated coronary artery significantly increased CBF (p < 0.001) (Figure 4B). No changes in heart rate, mean arterial pressure (Figure 4A), or cardiac rhythm were observed (Figures 4A, 4C, and 4D) and no differences in global LV function were detected (Figures 4E and 4F). There was a trend toward improved +dP/dt max and LV peak developed pressure when mitochondria were added to ADH compared with ADH alone; however, they did not reach statistical significance. Regional LV function, as measured by the proportion of segmental



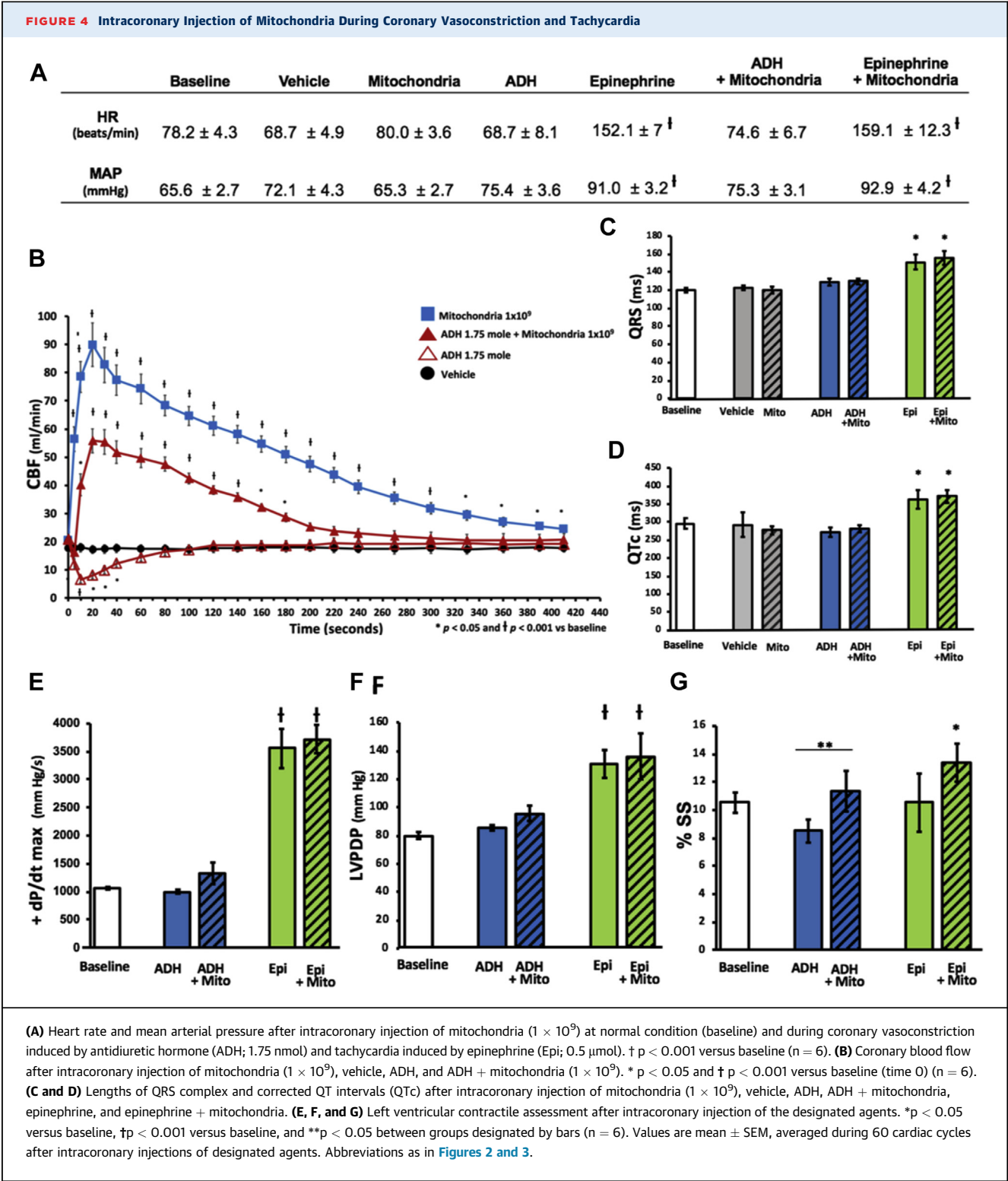
shortening, showed a modest increase on injection of mitochondria into the vasoconstricted LCA (11.3% ± 1.5% vs. 8.5% ± 0.8%; p = 0.017) (Figure 4G). Intracoronary injection of epinephrine produced significant tachycardia and systemic hypertension (Figure 4A). Mitochondrial injection in the epinephrine-treated coronary artery produced no changes in heart rate, mean arterial pressure, cardiac rhythm, or regional or global LV function (Figures 4A

to 4G). Evaluation of CBF in response to epinephrine with mitochondria was excluded from the analysis because the significant tachycardia and hypertension created by epinephrine causes a secondary increase in CBF and confounds the primary changes in CBF by mitochondria. To provide a positive control, a separate study was performed in which polystyrene microbeads (3, 10, and 150 μm, 1 × 10⁹ each) were injected separately

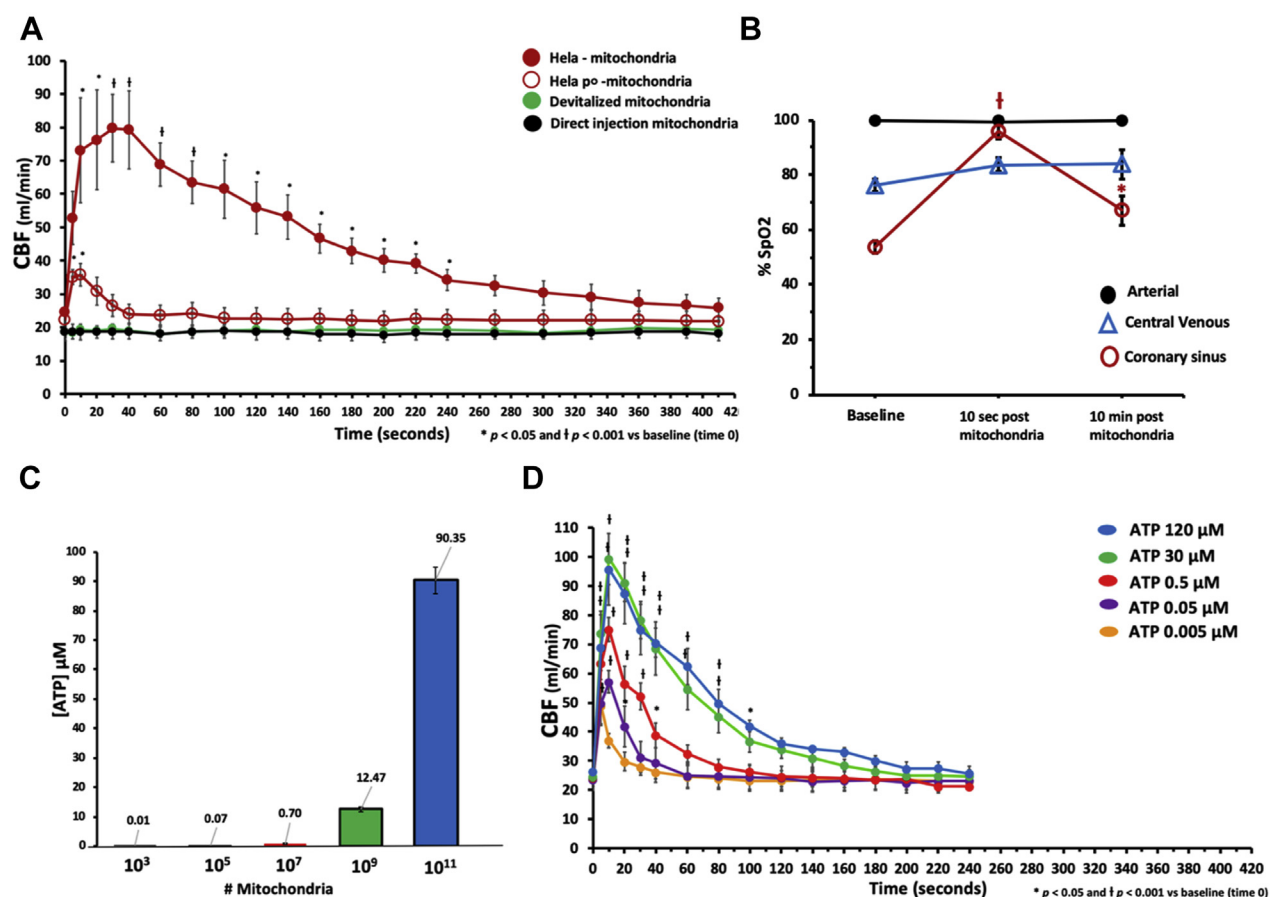
FIGURE 3 Coronary Patency and Coronary Blood Flow



(A) Representative coronary angiography of swine under baseline condition (Video 1) and (B) immediately after intracoronary injection of 1×10^9 mitochondria (Video 2). Transonic flow probe (arrows). (C) Continuous coronary blood flow (CBF) at the mid left anterior descending artery on intracoronary injection of vehicle and different concentrations of mitochondria ($n = 6$). (D) Comparisons of the area under the curve (AUC) of graph (C) using the trapezoidal rule from time 0 to 410 s, in milliliters per min $\times$ s. (E) CBF and (F) heart rate, mean arterial pressure, and left ventricular end-diastolic pressure on serial, intracoronary injections of mitochondria (1×10^9) every 5 min, 10 times. Arrows denote the times of mitochondrial injection. Values are mean $\pm$ SEM. * $p < 0.05$ and † $p < 0.001$ versus baseline (time 0) ($n = 5$). LAD = left anterior descending artery; LCX = left circumflex artery. Abbreviations as in Figure 2.



into the LCA ($n = 5$). The sizes of the microbeads were chosen to exceed the size ranges of mitochondria (0.5–1.0 μ m) (Supplemental Figure S1). Intracoronary injection of 3- μ m microbeads had no effect on hemodynamics or LV function (Supplemental Figure S2). In contrast, 10- and 150- μ m microbeads, which significantly exceed the size of the injected mitochondria, resulted in significant coronary

FIGURE 5 Coronary Blood Flow and Mitochondrial Respiration Capacity

(A) Coronary blood flow on direct myocardial injection of mitochondria at 10 different sites in close proximity to the left anterior descending artery (1×10^9 total; $n = 3$), intracoronary injection of devitalized mitochondria (1×10^9 ; $n = 4$), and mitochondria isolated from HeLa cells and from HeLa- p^0 cells ($n = 6$). * $p < 0.05$ and $\dagger p < 0.001$ versus baseline (time 0). (B) Percentage oxygen saturation (%SpO₂) of blood from the carotid artery (arterial), superior vena cava (central venous), and coronary sinus collected 10 s (during peak increase in coronary blood flow) and 10 min after intracoronary injection of mitochondria ($n = 4$). Values are mean $\pm$ SEM. * $p < 0.05$ and $\dagger p < 0.001$ versus baseline %SpO₂ within each group. (C) ATP content present in various concentrations of mitochondria. (D) Coronary blood flow on intracoronary injection of ATP alone, as measured in the various concentrations of mitochondria. * $p < 0.05$ and $\dagger p < 0.001$ versus baseline (time 0) ($n = 4$).

ATP = adenosine triphosphate; other abbreviations as in Figure 3.

occlusions and myocardial contractile failure (Supplemental Figure S2).

PHASE 2: EVALUATION OF INCREASE IN CORONARY BLOOD FLOW. The role of mitochondrial viability and respiration competence.

In contrast to intracoronary injection of mitochondria, there was no change in CBF associated with direct injection of mitochondria to the myocardium (Figure 5A) ($n = 3$). If myocardial oxygen consumption increased, it would also be reflected by the decrease of coronary sinus proportion venous oxygen saturation associated with a compensatory increase in CBF (27). On the contrary, coronary sinus proportion venous oxygen saturation

increased to near arterial levels immediately on intracoronary delivery of mitochondria, from $53.7\% \pm 2.4\%$ to $96.1\% \pm 1.3\%$ ($n = 4$; $p < 0.001$), concomitant with the increase in CBF (Figure 5B).

The role of mitochondrial viability and respiration competence in mitochondria-induced coronary vasodilation was investigated by intracoronary injection of devitalized mitochondria, HeLa and HeLa- p^0 mitochondria. Intracoronary injection of devitalized mitochondria (1×10^9) did not alter CBF ($n = 4$) (Figure 5A). Intracoronary injection of HeLa mitochondria, which are capable of oxidative phosphorylation, increased CBF from 24.6 ± 2.9 ml/min to 79.7 ± 8.1 ml/min ($n = 6$; $p < 0.001$) (Figure 5A). In

contrast, intracoronary injection of HeLa-p^o mitochondria, which are not capable of oxidative phosphorylation, had no effect on CBF (n = 6) (HeLa AUC 18,416.5 ± 2,204.4 vs. HeLa-p^o AUC 9,524.27 ± 1,230.1 ml/min × s; p = 0.005) (Figure 5A).

The role of ATP. To investigate the role of mitochondrial energy synthesis in coronary vasodilation, ATP content in the previously tested mitochondrial concentrations (1 × 10³, 1 × 10⁵, 1 × 10⁷, 1 × 10⁹, and 1 × 10¹¹) was determined and the corresponding doses of ATP alone were injected into the LCA (n = 4) (Figure 5C). Intracoronary injection of ATP in the absence of mitochondria increased CBF similarly to the corresponding concentration of mitochondria (Figure 5D). However, the duration of hyperemia was significantly shorter than that observed from intracoronary injection of mitochondria. For instance, the injection of 1 × 10⁹ mitochondria provided 6.5 ± 0.6 min of increase in CBF versus 3.3 ± 0.2 min when 30 μM of ATP (amount found in 1 × 10⁹ mitochondria) was injected alone (p < 0.001).

Signaling pathways of coronary vasodilation. Mitochondria-induced increase in CBF was unaffected by the inhibition of endothelium-derived pathways of coronary vasodilation (nitric oxide synthase and cyclooxygenase) (Figures 6A and 6B) (n = 4 each). Inhibition of the adenosine receptors and inhibition of K_{ATP} channels also had no effect on mitochondria-induced increase in CBF (Figures 6C and 6E) (n = 4 each). In contrast, inhibition of K_{IR} channels significantly attenuated mitochondria-induced increase in CBF from 97.8 ± 11.9 ml/min to 60.1 ± 8.4 ml/min (p = 0.018) and to 50.2 ± 7.6 ml/min (p = 0.012) at barium chloride concentrations 10 and 100 μM, respectively (Figure 6E) (n = 4).

PHASE 3: CARDIOPROTECTIVE EFFICACY OF MITOCHONDRIAL TRANSPLANTATION BY INTRACORONARY DELIVERY IN ISCHEMIA-REPERFUSION INJURY. Animals. There was no significant difference in animal body weight between the mitochondria group and the vehicle-only group (p = 0.133). One animal in the vehicle group died of refractory ventricular fibrillation at the onset of reperfusion.

Post-ischemic myocardial function. There were no differences in the pre-ischemic vital signs or LV contractility between the 2 groups (Figures 7A to 7G). With the onset of regional ischemia, significant depressions in LV contractility were observed in both the mitochondria group (n = 8) and vehicle-only group (n = 7) (Figures 7C to 7G), but no differences were observed between the groups. During reperfusion, all contractile measures were significantly higher in the mitochondria group (Figures 7C to 7G)

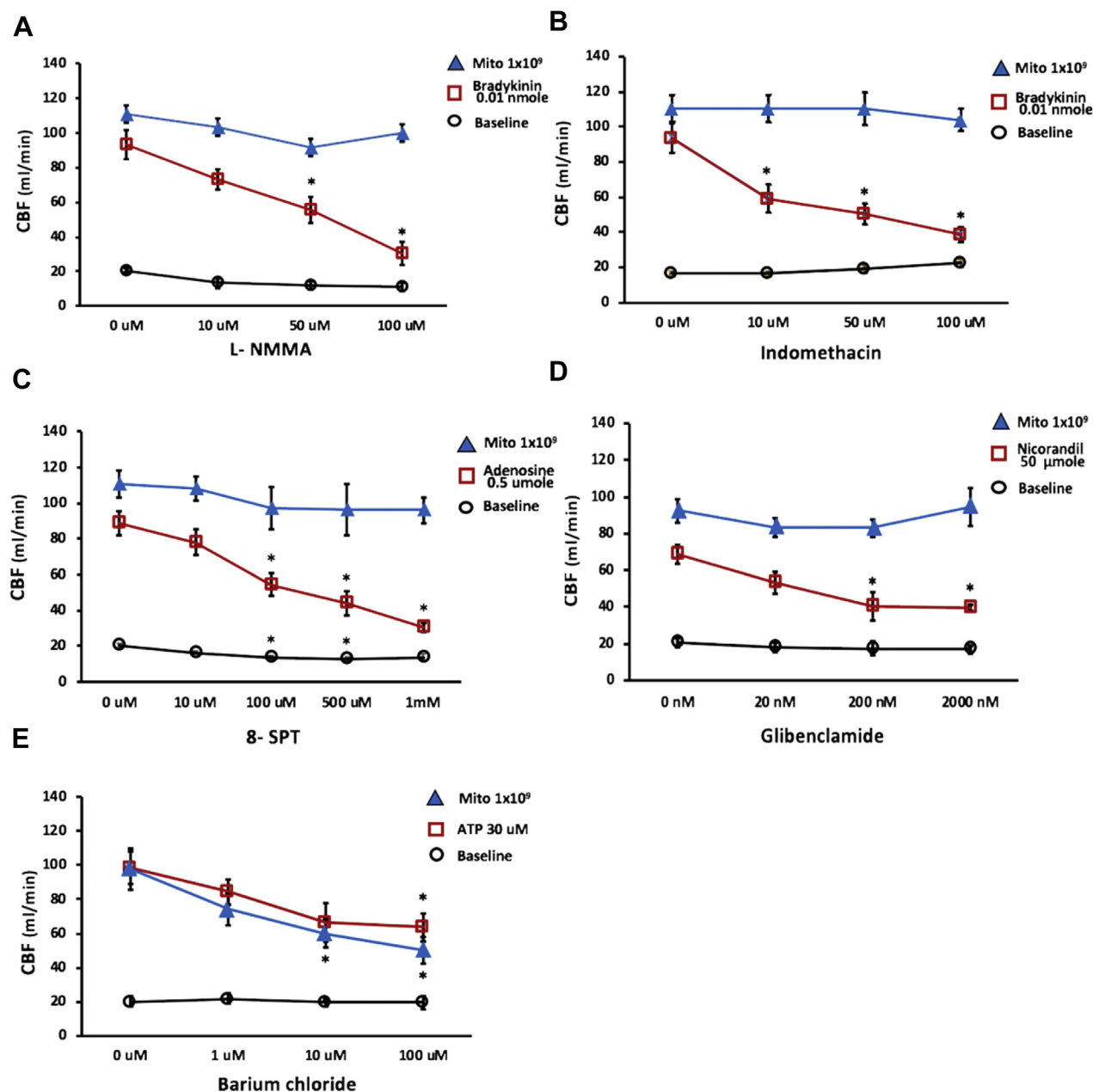
and returned to pre-ischemic levels by the end of the reperfusion period. Similarly, echocardiographic analyses showed superior LV function in the mitochondria group as measured by proportion of LV fractional shortening (23.8% ± 2.8% vs. 13.9% ± 1.2%; p = 0.004), proportion LV fractional area change (41.8% ± 2.9% vs. 28.6% ± 2.3%; p = 0.003), and proportion ejection fraction (EF) (47.9% ± 4.6% vs. 30.2% ± 2.3%; p = 0.003) at 2 h of reperfusion (Figures 8B to 8D, Videos 3 and 4).

Post-ischemic coronary blood flow. The mitochondria group exhibited significantly higher CBF throughout the reperfusion period compared with the vehicle-only group. Pre-ischemic CBF was 13.4 ± 1.9 ml/min and 11.1 ± 2.5 ml/min in the mitochondria group and vehicle-only group, respectively (Figure 8A) (p = 0.462). With the onset of ischemia, CBF decreased to near 0 ml/min in both groups. Immediately on reperfusion, reactive hyperemia was observed in both groups to peak at CBF levels of 76.0 ± 4.5 ml/min in the mitochondria group and 67.4 ± 16.5 ml/min in the vehicle-only group. However, 19.2 min post-reperfusion, mean CBF remained significantly higher in the mitochondria group, and this increase was present until the end of the 120-min reperfusion period (AUC 1,329.5 ± 277.9 vs. 550.2 ± 119.1 ml/min × s; p = 0.042) (Figure 8A).

Infarct size. There was no difference in the mean area at risk (proportion of LV mass) between the mitochondria group and the vehicle-only group (37.4 ± 1.9 vs. 35.4 ± 2.7; p = 0.343). Infarct size (proportion of area at risk) was significantly reduced in the mitochondria group (7.3% ± 1.1% vs. 38.6% ± 2.7%; p < 0.001) (Figures 8E and 8F).

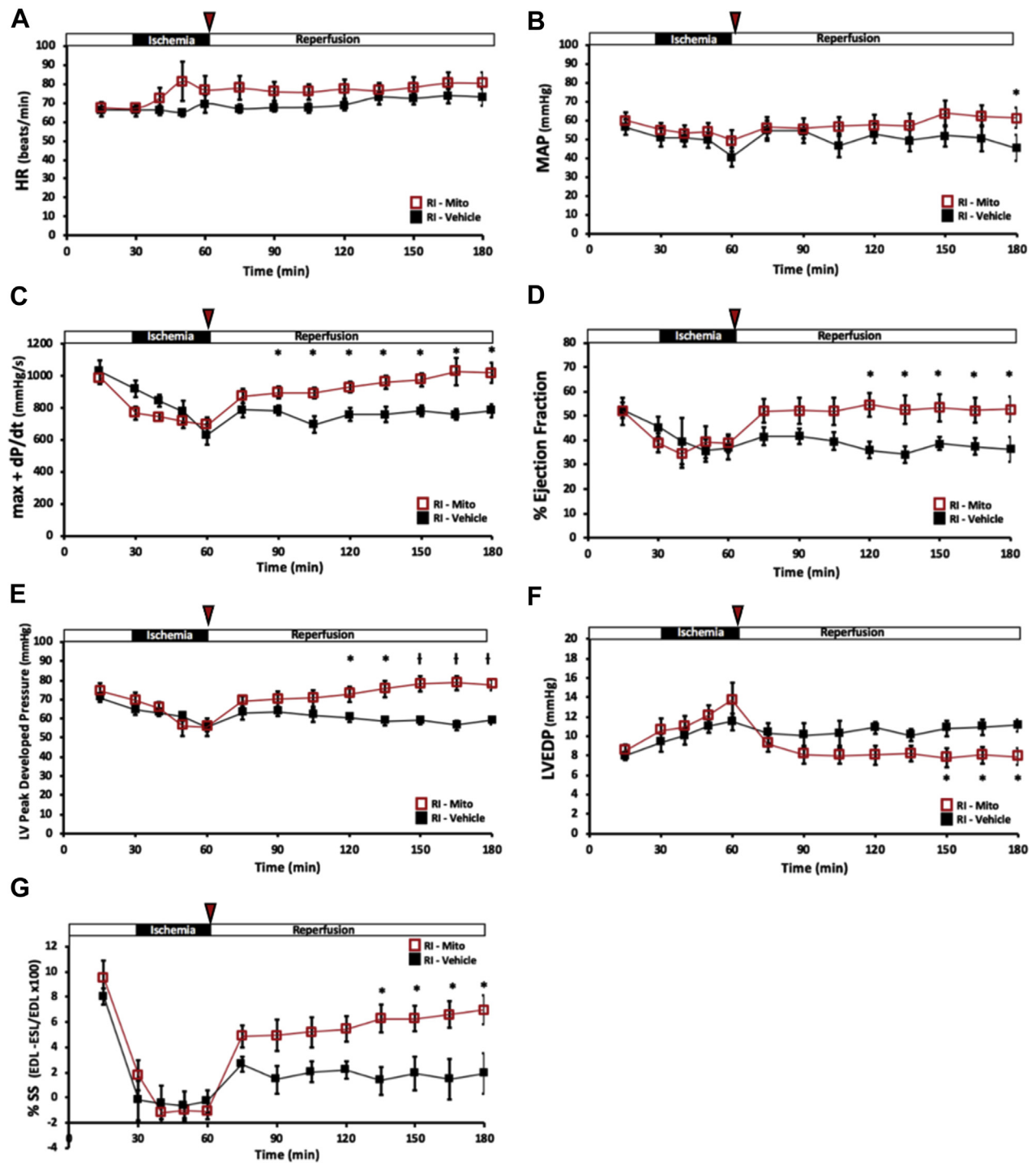
DISCUSSION

In our previous studies, transplantation of mitochondria was performed by direct tissue injection with a 28-gauge-needle syringe (6,8-10,14,15). This technique allows specific delivery of the donor mitochondria to a localized area of the myocardium. Although effective, it requires open-heart access, potential manipulation of the heart, and multiple injections. We have previously reported the histologic quantification of radioactive, iron oxide-labeled, human mitochondria transplanted to isolated perfused rabbit hearts through intracoronary delivery (11). Similar to our present findings, positron emission tomographic imaging of these isolated hearts showed global distribution of radioactive signal throughout the entire organ, whereas histologic analysis showed mitochondria within blood vessels (25.7%), cardiomyocytes (23.6%), and the

FIGURE 6 Mechanism of Mitochondria-Induced Coronary Vasodilation

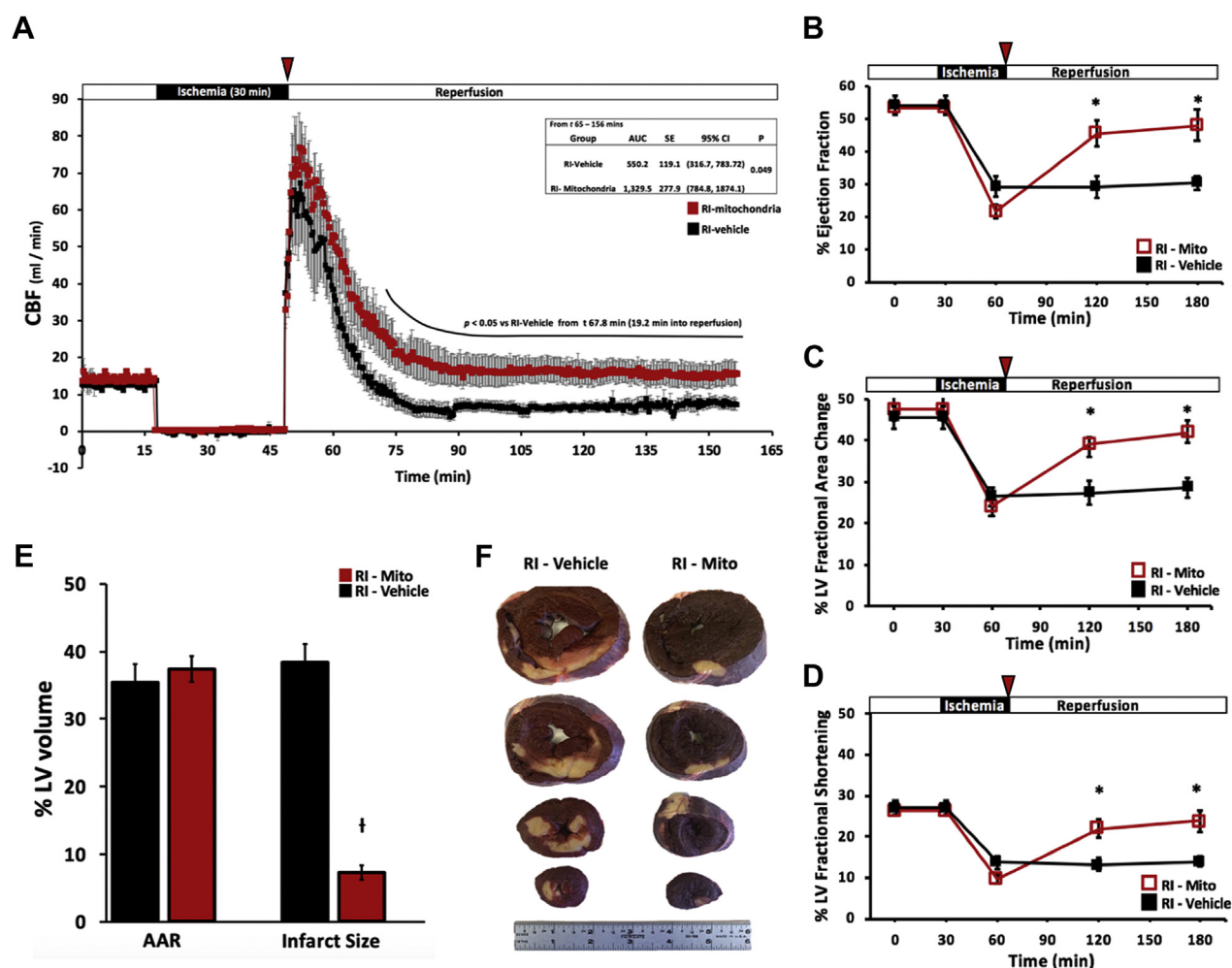
(A) CBF on intracoronary injection of mitochondria (1×10^9) after pretreatment with increasing concentrations of nitric oxide synthase inhibitor nitro-monomethyl L-arginine (L-NMMA). CBF after bradykinin injection (nitric oxide synthase activator, 0.01 nmol) shows positive inhibition of nitric oxide synthase ($n = 4$). Baseline indicates CBF after 20 min of pretreatment with L-NMMA before intracoronary injection of mitochondria or bradykinin. (B) CBF on intracoronary injection of mitochondria after pretreatment with increasing concentrations of cyclooxygenase inhibitor indomethacin. CBF after bradykinin injection (cyclooxygenase activator, 0.01 nmol) shows positive inhibition of cyclooxygenase ($n = 4$). (C) CBF on intracoronary injection of mitochondria after pretreatment with increasing concentrations of adenosine receptor inhibitor 8-*p*-sulphophenyl theophylline (8-SPT). CBF after adenosine (0.5 μ mol) injection shows positive inhibition of adenosine receptor ($n = 4$). (D) CBF on intracoronary injection of mitochondria after pretreatment with increasing concentrations of K_{ATP} -channel inhibitor glibenclamide. CBF after nicorandil injection (K_{ATP} -channel activator, 50 μ mol) shows positive inhibition of K_{ATP} channels ($n = 4$). (E) CBF on intracoronary injection of mitochondria after pretreatment with increasing concentrations of K_{IR} -channel inhibitor barium chloride. CBF after ATP injection (K_{IR} -channel activator, 30 μ M) shows positive inhibition of K_{IR} channels ($n = 4$). Values are $\pm$ SEM; * $p < 0.05$ versus CBF in the absence of inhibitor within the same group. Abbreviations as in Figures 3 and 5.

FIGURE 7 Myocardial Function After Intracoronary Mitochondrial Transplantation in Regional Myocardial Ischemia-Reperfusion Injury



(A) Heart rate, (B) mean arterial pressure, (C) max + dP/dt (mm Hg), (D) proportion ejection fraction, (E) left ventricular peak developed pressure (mm Hg), (F) left ventricular end-diastolic pressure (mm Hg), and (G) proportion segmental shortening at the end of systole in the vehicle-only group (RI-Vehicle) and mitochondria group (RI-Mito) at pre-ischemia, during 30 min of regional ischemia and 120 min of reperfusion. Arrowheads denote the time of intracoronary injection of either vehicle or mitochondria. * $p < 0.05$ and † $p < 0.001$ between the 2 groups. Abbreviations as in Figure 2.

FIGURE 8 Coronary Blood Flow and Tissue Survival After Intracoronary Mitochondrial Transplantation in Regional Ischemia-Reperfusion Injury



(A) CBF at the left anterior descending artery distal to temporary occlusion in the vehicle group (RI-Vehicle) and mitochondria group (RI-Mito). $p < 0.05$ between 2 groups from 67.8 min (19.2 min into reperfusion) to the end of reperfusion (120 min). Areas under the curve are compared between the 2 groups from 65 min after reactive hyperemia, in which mean CBF exhibited a statistically significant difference. (B, C, and D) Echocardiographic analysis of left ventricular (LV) function analyzed from short-axis view and M-mode tracings at the midpapillary level. Values are mean $\pm$ SEM; * $p < 0.05$ versus RI-Vehicle (Videos 3 and 4). (E) Area at risk (proportion LV volume) and infarct size proportion of area at risk) after 120 min of reperfusion. † $p < 0.001$ versus RI-Vehicle. Arrowheads denote the time of intracoronary injection of either vehicle or mitochondria. (F) Representative photograph of hearts stained with triphenyl tetrazolium, showing infarct sizes in RI-Vehicle and RI-Mitochondria groups. Abbreviations as in Figure 3.

interstitium (11). Distribution of mitochondria appeared scattered but uniform, consistent with global dispersal by intravascular delivery, as opposed to clustered appearance when delivered via localized direct injections into the myocardium (11). Our previous studies also suggest that the number of mitochondria needed for cardioprotection is not a function of the absolute number of mitochondria transplanted; for example, 2×10^5 to 2×10^8 mitochondria per gram of tissue had the same extent of improvements in infarct sizes, regional segmental

shortening, and increased total tissue ATP content in the area at risk (6,8,9).

In the present study, we focused our attention on demonstrating the systemic distribution of mitochondria when delivered via the coronary arteries. We first evaluated the uptake and biodistribution of mitochondria by intracoronary delivery, using ^{18}F -rhodamine-6G, which specifically labels actively respiring mitochondria (16). Positron emission tomographic imaging demonstrated that intracoronary delivery distributed mitochondria specific to the

vascular supply of LCA. Minor signal was detected in the descending aorta, likely because of forward flow of blood from the ascending aorta as mitochondria were injected into the LCA. The tracer signal was not detected in other organs despite the injection of much higher concentrations of mitochondria than the therapeutic dosage. We did not demonstrate long-term viability of the donor mitochondria in this nonsurvival study. We have previously demonstrated the presence of transplanted mitochondria in the area at risk in swine hearts 28 days after injection of vehicle containing mitochondria (10). These regions displayed higher total tissue ATP content than the experimental control area at risk that received vehicle-only injections (10).

The mechanisms of vascular extravasation of mitochondria are beyond the scope of the current investigation and remain to be fully elucidated. However, the rapidity of mitochondria transport to cardiac cells is likely to involve mechanisms similar to those involved in bacterial or viral uptake (28-30). Support for such pathways may be seen in our studies showing the rapid uptake of mitochondria, using ^{18}F -rhodamine-6G-labeled mitochondria (11), and that reported by Bomberger et al. (31) demonstrating the rapid cellular uptake of rhodamine-R18-labeled outer membrane vesicles, as well as those by others showing rapid uptake of intact extracellular vesicles (28,29). Moreover, numerous studies demonstrating cellular transformations of exogenous mitochondria suggest that the process requires both the physical and functional integrity of mitochondria. We previously showed that only intact, respiration-competent mitochondria are taken up by cardiac cells (11,12). Kesner et al. (29) also reported that cellular uptake of exogenous mitochondria is inhibited by disruptions in the mitochondrial outer membrane. These results correlate with our previous findings demonstrating the inability of nonviable mitochondria, mitochondrial fractions, mitochondrial deoxyribonucleic acid, ribonucleic acid, and exogenous adenosine diphosphate and ATP to provide protection to the ischemic heart (8,14).

A major safety concern of intracoronary injection of particles is the risk of microvascular obstruction. Concerns in regard to intracoronary infusion of particles $>10\text{ }\mu\text{m}$ in diameter have been raised (32), whereas others have reported various degrees of safety with larger agents such as mesenchymal stem cells ($\approx 20\text{ }\mu\text{m}$) which may exceed the diameter of some resistance arterioles (33-35). In contrast, mitochondria are $0.3 \pm 0.1\text{ }\mu\text{m}$ in diameter (Supplemental Figure S1) in both swine and in humans (11,13), which is smaller than the diameter of the smallest capillaries

present in human and swine hearts ($5\text{-}10\text{ }\mu\text{m}$) (35). According to our results, intracoronary injection of mitochondria at concentrations of 1×10^3 to 1×10^{11} has no adverse effects on coronary patency or cardiac function. Mitochondria were also safely injected into severely constricted coronary arteries as well as under hemodynamic stresses of significant tachycardia and hypertension, all of which often accompany various pathologic conditions of the heart. The safety of intracoronary infusion of mitochondria is further corroborated by adverse response to intracoronary injection of microbeads (10 and $150\text{ }\mu\text{m}$) that are larger than the diameters of the small and midsize coronary arterioles of swine (36), resulting in significant coronary occlusion, arrhythmia, and contractile failure, none of which were observed with intracoronary injections of mitochondria.

To our knowledge, this is the first study to provide evidence that intracoronary infusion of mitochondria significantly increases CBF. This effect on CBF was immediate and concentration dependent, with maximal hyperemia achieved by intracoronary injection of 1×10^9 mitochondria. The duration of hyperemia by mitochondria ($6.5 \pm 0.6\text{ min}$) is significantly higher than that of many of the mainstream pharmacologic coronary vasodilators such as papaverine ($\approx 50\text{ s}$) and adenosine ($\approx 20\text{ s}$) (37), and the hyperemia was safely extendable by serial injections. Furthermore, intracoronary injection of mitochondria was able to entirely reverse the vasoconstrictions induced by a potent coronary vasoconstrictor.

Our results suggest that ATP is at least partly responsible for mitochondria-induced hyperemia because the increase in CBF was achievable only through the delivery of intact, respiration-competent mitochondria. ATP has been reported to be a potent dilator of coronary circulation (38-40). This is further corroborated by our finding that mitochondria-induced hyperemia was attenuated only by the inhibition of the K_{IR} channels and unaffected by the inhibition of nitric oxide synthase, cyclooxygenase, or adenosine receptor. These findings are consistent with previous reports that ATP-mediated coronary vasodilation is largely independent of endothelium-derived pathways or the breakdown of ATP to adenosine (40), and that K_{IR} channel is involved in ATP-mediated vasodilation (23,41). The inhibition of K_{IR} channels only partly abolished the vasodilatory effect by mitochondria, suggesting the presence of additional and yet-unidentified mechanisms.

CBF in response to intracoronary injection of ATP alone paralleled that of the corresponding mitochondrial concentrations in the magnitude of increase in CBF; however, the durations of hyperemia

were significantly shorter. One possible explanation for these findings may be related to the short half-life of exogenous ATP in blood (0.5 to 1.5 s) (42), rendering it undesirable for clinical application (43). Intact mitochondria with active electron transport chain and ATP synthesis may continuously renew ATP as they are infused in the coronary arteries, leading to the prolongation of the vasodilatory effect of ATP. Support for such a mechanism comes from our previous studies demonstrating increased cellular ATP content and ATP synthesis after mitochondrial transplantation (9,11-13). Although further studies are required to delineate the full mechanism, our results implicate the sustained production of ATP as a key mediator and the activation of K_{IR} channels as a downstream pathway of mitochondria-induced coronary vasodilation.

Our results demonstrate strong cardioprotective efficacy of intracoronary mitochondrial transplantation in myocardial ischemia-reperfusion injury by improving post-ischemic function, perfusion, and infarct size. We used a model of temporary coronary occlusion followed by mitochondrial injection at reperfusion because, in current practice, most patients with acute myocardial ischemia undergo reperfusion by emergency coronary catheterization, which would also be an opportune time to administer mitochondria.

Our results also show that the increase in post-ischemic CBF was sustained throughout the 120 min of reperfusion in the mitochondria hearts compared with vehicle hearts. The increase in CBF is an advantageous phenomenon unique to intracoronary injection and absent when mitochondria are directly injected into the heart muscle. Although the improvement of CBF is therapeutically beneficial, our previous studies have shown that direct injection and intravascular delivery provide similar improvements in post-ischemic functional recovery and infarct size (8,9,11). This indicates the presence of a primary cardioprotective process at the level of the tissue that is separate and successive to the improved CBF after intracoronary infusion of mitochondria. At present, our studies have found that direct injections of mitochondria lead to increase in the ATP content of the recipient tissue, upregulation of proteomic pathways for the mitochondrion and precursor metabolites, reduction of inflammatory mediators, upregulation of antiapoptotic markers, and replenishment of damaged mitochondrial deoxyribonucleic acid (8-10). It is therefore reasonable to suspect, at least at the tissue level, that intracoronary delivery of mitochondria leads to the same changes as direct muscle injection once they have reached the tissue.

Nevertheless, other investigations have shown benefits of coronary vasodilators in improving microvascular dysfunction and impaired perfusion that are present in ischemic injury (44,45). In the setting of ischemia-reperfusion, mitochondria resulted in a post-peak CBF that was higher throughout the entire reperfusion period compared with a transient effect of approximately 7 min in the nonischemic hearts. Although the full mechanism remains to be elucidated, the biochemical milieu of the ischemic heart is markedly different from that of the normal heart. We speculate that intracoronary delivery of mitochondria may offer the dual benefit of counteracting impaired tissue perfusion at the level of the coronary arteries and rescuing metabolic and inflammatory pathways at the tissue level. The rescue of cardiomyocytes from both approaches may further reduce the production of various vasoconstrictive signals (8,9), producing a synergistic and cascading improvement to CBF, myocardial function, and infarct size.

Intracoronary use of mitochondria provides a variety of possible applications for both angiographic and surgical therapies by exploiting its multifactorial rescue of myocellular damage (9,14), coronary vasodilation, and the versatility of the minimally invasive catheter-based delivery. Mitochondria can be effectively delivered by bolus injections to the heart by rapid continuous-flow infusions rather than by the stop-reflow technique, which involves the temporal coronary occlusions used during certain cell therapies such as mesenchymal stem cells, which carry the risk of arrhythmia and myocardial injury (46). Mitochondria may also serve as an adjunct to percutaneous coronary intervention treatments for acute myocardial ischemia, in place of current vasodilatory drugs investigated for the prevention and treatment of post-ischemic microvascular dysfunction ("no reflow"), including adenosine (47), nitroprusside (48), verapamil (49), and nicorandil (50), without the risks of the pharmacologic adverse effects. Although validation in humans is required, mitochondria may offer a safer profile while providing near-maximum vasodilatory capacity of the coronary vasculature (51). Mitochondria may also be used during coronary catheterization in subacute settings such as myocardial stunning or hibernation because contractile improvements have been shown from transplantations 2 to 15 days after ischemic injury in humans (15).

STUDY LIMITATIONS. Certain limitations of our study must be considered when these results are interpreted. First, in our investigation of safety, we used only healthy adult swine. Although we have attempted to mimic pathologic cardiac physiology with vasoactive drugs, our use of healthy subjects

may underestimate potential complications associated with additional cardiopulmonary dysfunction such as heart failure, respiratory distress, and/or other organ dysfunction. We have also not yet obtained analyses of additional organ function (e.g., kidneys, liver) after intracoronary injection of mitochondria. Although previous animal and human data have not detected changes in systemic inflammatory markers, or respiratory or renal function, randomized controlled clinical trials addressing short-term and long-term outcomes are required.

CONCLUSIONS

In conclusion, mitochondrial transplantation by intracoronary delivery to the myocardium is safe and efficacious, with strong vasodilatory capacity, which translates to significant therapeutic efficacy in treating myocardial ischemia-reperfusion injury. The capacities of metabolic restoration, cardiomyocyte salvage, and coronary vasodilation may be harnessed to produce therapeutic synergy, with the present findings serving as a preclinical platform to help optimize human application across the clinical spectrum of ischemic heart disease and coronary regulation.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: The use of healthy mitochondria to treat ischemia-reperfusion injury has been successfully applied in various animal models in the heart, kidneys, liver, lung, and brain. The therapeutic benefit of mitochondrial transplantation in humans was recently described in pediatric cardiac ischemia-reperfusion patients, using direct myocardial injection into the myocardium.

TRANSLATIONAL OUTLOOK: This study supplies the pre-clinical validation of safety and efficacy of intracoronary delivery of mitochondria, which significantly widens the application and potential usage of mitochondrial transplantation. Controlled, multicenter, prospective studies are warranted in pediatric and adult cardiac patients to confirm safety and efficacy.

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KEY WORDS ischemia-reperfusion injury, mitochondria, mitochondrial transplantation, myocardial protection

APPENDIX For supplemental figures and videos please see the online version of this paper.