

Mitochondrial Respiratory Capacity is Restored in Hibernating Cardiomyocytes Following Co-Culture with Mesenchymal Stem Cells

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Laura L. Hocum Stone^{1,2}, Erin Chappuis^{1,2}, Maribel Marquez³,
Edward O McFalls^{1,4}, Rosemary F. Kelly^{1,2}, and Tammy Butterick^{1,5}

Abstract

Hibernating myocardium is a subset of ischemic cardiac disease characterized by viable but dysfunctional tissue. Standard treatment for hibernating myocardium is coronary artery bypass graft, which reduces arrhythmias and improves survival but does not fully restore function, presenting a gap in currently available treatments. Large animal studies of hibernating myocardium have identified impaired mitochondrial dynamics as a root cause of persistent cardiac dysfunction despite surgical revascularization. This study presents a novel *in vitro* model of hibernating myocardium cardiomyocytes to study active mitochondrial respiration in hibernating myocardium cells, and to test the paracrine effect of mesenchymal stem cells on impaired mitochondrial function. Exposure of cardiomyocytes to hypoxic conditions of 1% oxygen for 24 hours resulted in a phenotype consistent with hibernating myocardium cardiac tissue, including decreased respiratory capacity under high work states, decreased expression of mitochondrial proteins, and preserved cellular viability. Co-culture of hibernating myocardium cardiomyocytes with mesenchymal stem cells restored mitochondrial respiratory function, potentially via an increase in proliferator-activated receptor gamma coactivator 1-alpha-driven mitochondrial biogenesis. Co-culture treatment of hibernating myocardium cardiomyocytes with mesenchymal stem cells shows improvement in both mitochondrial function and ATP production, both of which are critical for effectively functioning cardiac tissue. These results suggest that mesenchymal stem cell therapy as an adjunct treatment to revascularization may address the current gap in treatment for hibernating myocardium patients.

Keywords

Hibernating myocardium, mesenchymal stem cells, mitochondrial respiration, peroxisome proliferator-activated receptor gamma coactivator 1-alpha

Introduction

Although the mortality rate associated with cardiac disease has decreased in recent years, it remains one of the leading causes of death globally¹. Ischemic cardiac disease presents as a large clinical spectrum, yet pre-clinical research in this area focuses largely on myocardial infarction (MI). MI is characterized by the presence of infarct tissue that cannot be rescued. At the other end of the spectrum is hibernating myocardium (HM), a subtype of cardiovascular disease that has potential for full or partial recovery. HM is defined as chronic ischemia due to reduced coronary artery blood flow that does not result in cell death, yet has decreased regional function, impaired ability to respond to increased work demands, and increases risk of arrhythmia². Although HM

¹ Research Service, Minneapolis VA Health Care System, USA

² Department of Surgery, University of Minnesota, USA

³ Department of Physiology, Cardiovascular Center, Genomic Sciences and Precision Medicine Center, Medical College of Wisconsin, USA

⁴ Cardiology Division, Minneapolis VA Health Care System, USA

⁵ Department of Food Science and Nutrition, University of Minnesota, USA

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Corresponding Author:

Laura L. Hocum Stone, Minneapolis VA Health Care System, Building 70, Research 3M-118, 1 Veterans Drive, Minneapolis, MN 55417, USA.
Email: stone337@umn.edu



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tissue is viable, as noted by increased glucose uptake relative to oxygen consumption, standard treatment with coronary artery bypass graft (CABG) does not result in full functional recovery³, but does reduce the risk for future cardiac events and decrease mortality⁴.

Our group has a well-established swine model of HM that is amenable to CABG treatment, providing an animal model that closely mimics clinical HM cases⁵. This model of HM has allowed us to characterize proteomic changes in HM, identifying that the mitochondrial proteome is dysregulated in HM tissue, as exhibited by decreased expression of electron transport chain (ETC) complexes, mitochondrial fusion proteins, and regulators of mitochondrial biogenesis^{6–8}. These indicators of mitochondrial dysfunction are not restored to normal following restoration of blood flow with CABG^{9,10}. In addition, electron micrographs of this tissue show the mitochondria are small, segmented, and disorganized¹⁰. These data suggest the persistent mitochondrial dysfunction in HM despite revascularization inhibits full functional recovery.

To address this gap in treatment for HM, additional therapies are needed to address mitochondrial dysfunction and optimize myocardial recovery. HM presents a unique target for stem cell therapy as it is fully viable but dysfunctional cardiac tissue with the potential to be rescued. Mesenchymal stem cells (MSCs) are an ideal candidate for treatment, as previous studies have shown they have the potential to increase in peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1 α) signaling, which mediates mitochondrial biogenesis¹¹, suggesting a possible therapeutic mechanism for this mitochondrial-driven disease.

This study presents a novel *in vitro* model of HM in cardiomyocytes that exhibits preserved viability and decreased mitochondrial function in response to chronic, low-grade ischemia, mimicking the functional phenotype of HM hearts. This model allows, for the first time, an investigation of mitochondrial respiratory dynamics in HM as well as their response to mesenchymal stem cell therapy.

Materials and Methods

Isolation of Bone Marrow-Derived MSCs

MSCs were isolated as previously described¹². Briefly, 2 CPT (BD Vacutainer, San Jose, CA, USA) tubes containing swine bone marrow were collected aseptically and centrifuged to isolate the mononuclear fraction. Mononuclear cells were plated in complete growth media: Advanced Dulbecco's Modified Eagle's Medium (ADMEM) + 10% fetal bovine serum (FBS) (Hyclone, Logan, Utah, USA) + 1X Glutamax + 1X Pen/Strep (ThermoFisher, Waltham, MA, USA); media were exchanged daily for the first 3 days to isolate MSCs by adherent growth. Following isolation, the adherent cells were phenotyped for MSC markers by flow cytometry, confirming that MSCs did not express CD45 (leukocyte common antigen-45) and positively expressed the

MSC markers CD90 and CD105 (stem cell surface cluster of differentiation makers) (see Supplemental methods).

H9C2 Cardiomyocyte Culture

H9C2 rat heart myoblasts (American Type Culture Collection [ATCC] #CRL-1446, Manassas, VA, USA) were purchased and cultured in 1X Dulbecco's Modified Eagle's Medium [DMEM] + 10% FBS and 1X Pen/Strep (ThermoFisher, Waltham, MA, USA).

Induction of Hypoxia

H9C2 cells were plated in a six-well plate at 2×10^5 cells per well and allowed to grow in normoxic conditions for 24 hours (5% CO₂, 20% O₂, 37°C). MSCs were grown at 1.5×10^5 cells per well in a Corning Costar Transwell Cell Culture Insert (Corning, NY, USA) (see Supplement for details) and allowed to grow in normoxic conditions for 24 hours prior to transwell co-culture with H9C2 cells. Hypoxia was induced using the Evos FL Auto Onstage Incubator (ThermoFisher, Waltham, MA, USA) by exposing them to 37°C, 5% CO₂, and 1% O₂ conditions for 24 hours. Reoxygenation conditions consisted of moving cells that had been in hypoxia conditions for 24 hours to normoxic conditions for 24 hours.

Viability and Cell Counting

Cell viability was determined by trypan blue exclusion and quantification using the Countess II automated cell counter (ThermoFisher, Waltham, MA, USA).

Measurement of Intracellular Oxygen

Cellular hypoxia was confirmed by live-cell staining with ImageIT Hypoxia reagent (ThermoFisher, Waltham, MA, USA), which fluoresces when oxygen levels fall below 5%, and fluorescence intensity increases as oxygen concentration decreases. Hypoxia was quantified by measuring fluorescence intensity (excitation/emission 490/610 nm) using the EVOS FL Auto (ThermoFisher).

Measurement of Mitochondrial Respiration

Mitochondrial respiration was evaluated using the Agilent Seahorse XF Cell Mito Stress Test (Agilent, Santa Clara, CA, USA). Then 96-well plates were seeded with 8000 H9C2 cells per well and exposed to either normoxic or hypoxic culture conditions for 24 hours, with or without subsequent reoxygenation and/or MSC co-culture. MSC transwells were removed prior to measurement of oxygen consumption rate (OCR) in the co-culture groups to ensure respiratory measurements reflected H9C2 respiration alone. The Mito Stress Test to measure OCR was performed according to manufacturer protocols, using the following compound concentrations: 1 μ M oligomycin; 2 μ M

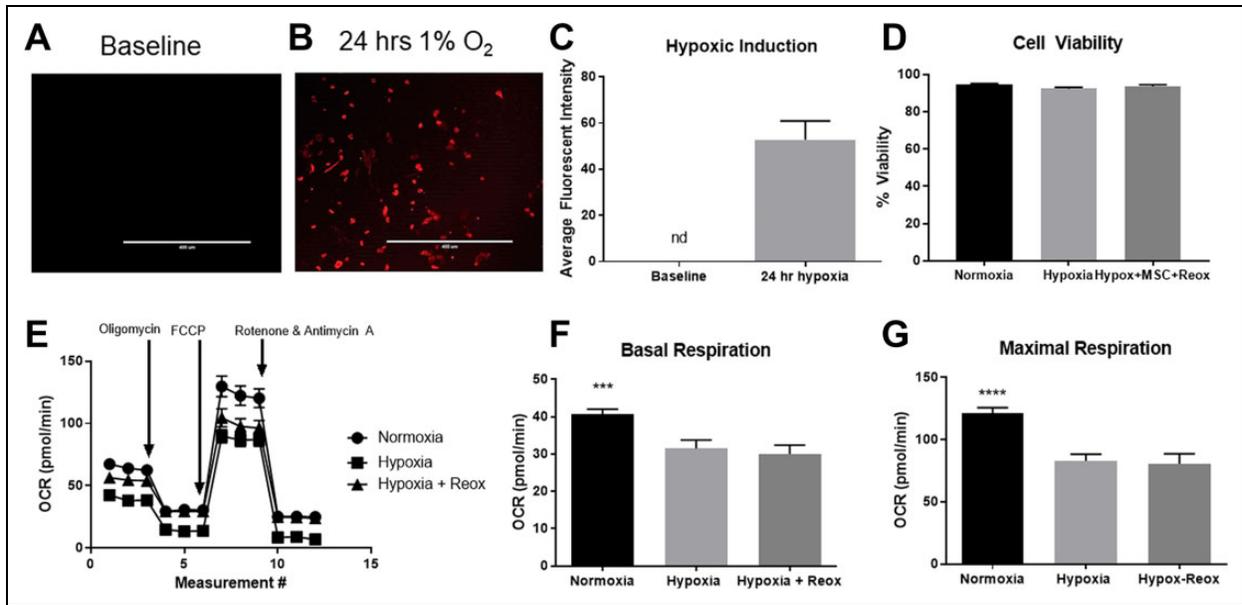


Figure 1. Cardiomyocyte exposure to 24 hours of 1% oxygen mimics the cellular phenotype of hibernating myocardium (HM). Measurement of cellular hypoxia at (a) baseline and (b) 24 hours following exposure to hypoxic conditions. (c) Quantification of hypoxia by measurement of fluorescent intensity ($n=4$; $p=0.0076$; nd=not detectable). (d) Cell viability was unchanged following exposure to 24 hours of hypoxia ($n=4$). (e) Measurement of oxygen consumption rate (OCR) indicated impaired respiratory capacity following hypoxia that was not restored following reoxygenation. Basal (f) and maximal (g) respiration was significantly reduced following hypoxia and was not restored following reoxygenation ($p=0.0005$ and $p<0.0001$, respectively).

trifluoromethoxy carbonylcyanide phenylhydrazide (FCCP); 0.5 μ M rotenone/antimycin A.

Real-time Polymerase Chain Reaction

Total RNA was isolated from the H9C2 cells from each condition using the QIAGEN RNeasy Mini Kit (#74104, Germantown, MD, USA) and were analyzed by Arq Genetics (Bastrop, Tx, USA), and all gene transcripts were normalized to normoxic samples using methodology previously described in Duffy et al¹³. See Supplement for primer information.

Western Blot

Total protein from nuclear fractions were isolated using a commercially available kit (Pierce Thermo Scientific) as described in the manufacturer's protocol. Nuclear protein concentrations were determined using a standard bicinchoninic acid (BCA) assay. Nuclear fractions were run in denatured and reduced conditions using equal amounts of protein on a 10% tris-glycine gel and transferred to a nitrocellulose membrane using the semi-dry TransBlot Turbo system (Bio-Rad). Membranes were probed with a primary PGC1 α antibody (ab54481, Abcam; 1:1000), and incubated with a near-infra-red secondary antibody (LiCor) for detection with the LiCor Odyssey Imager. Band density was quantified using Image Studio software (LiCor) and normalized to total protein (REVERT total protein stain, LICOR).

Statistics

All data are presented as mean \pm SEM. Differences between the means of multiple groups were compared using two-way analysis of variance with Tukey's test for multiple comparisons using GraphPad Prism (La Jolla, CA). A p value < 0.05 was considered significant.

Results

Cardiomyocytes exposed to 24 hours of hypoxia (1% oxygen) maintain viability but have impaired mitochondrial function. Hypoxia was confirmed by measurement of fluorescence intensity with Image IT Hypoxia dye (52.79 ± 8.2 vs 0.006 ± 0.004) (Figure 1(a)-(c)), and viability was quantified by trypan blue exclusion (Figure 1(d)) showing no increase in cell death under the hypoxic protocol. Following 24 hours of hypoxia, cardiomyocytes exhibited decreased basal (40.67 ± 1.4 vs 34.52 ± 2.24) and maximal respiration (121.06 ± 4.75 vs 82.95 ± 5.38) (Figure 1(e)-(g)). Following 24 hours of reoxygenation, measurements of basal (37.53 ± 2.78 vs 30 ± 2.41) and maximal (104.77 ± 7.9 vs 80.52 ± 8.21) respiration remain impaired. These characteristics of impaired function and maintained viability following the outlined hypoxic conditions provide an *in vitro* model consistent with the mitochondrial adaptations seen in HM.

Hibernating cardiomyocytes co-cultured with bone marrow-derived MSCs in a transwell system to prevent cell-cell contact and facilitate paracrine signaling during the reoxygenation period exhibited increased basal (30 ± 2.41 vs

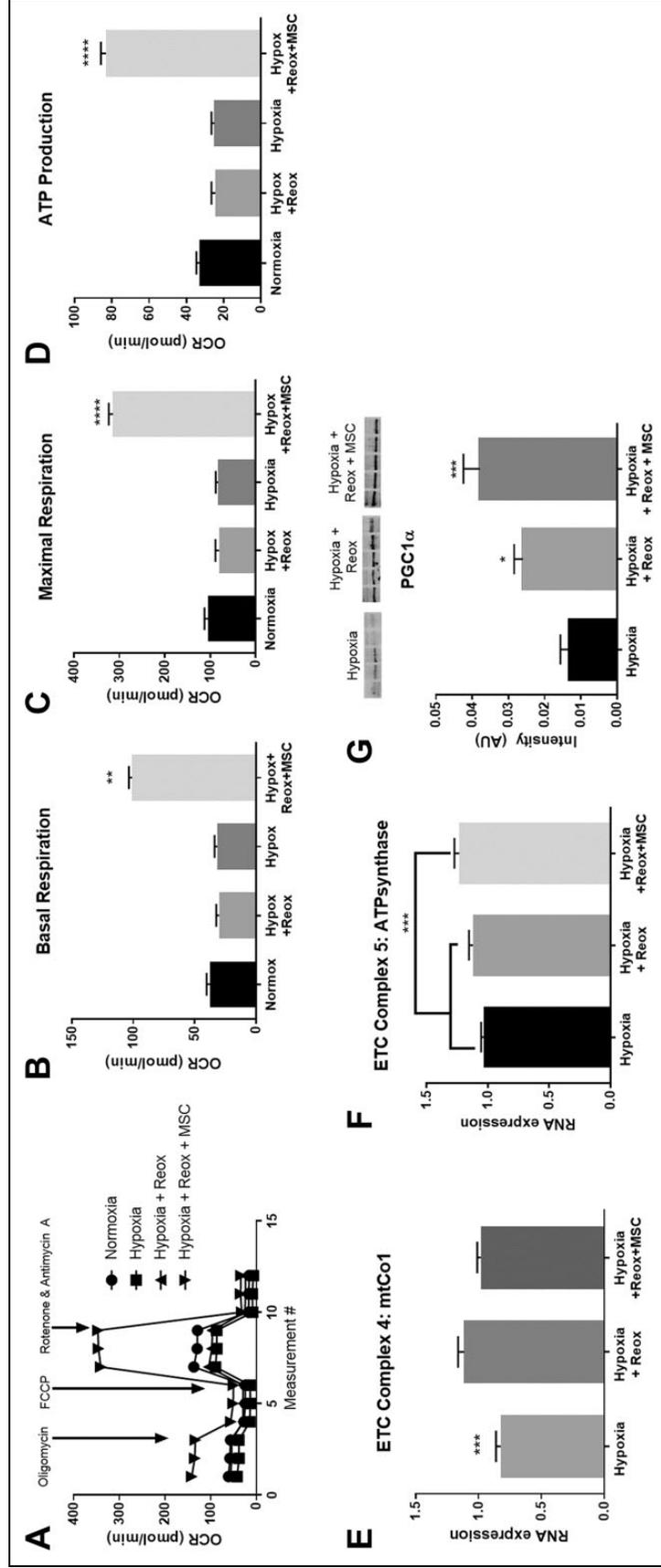


Figure 2. Transwell co-culture of mesenchymal stem cells (MSCs) during reoxygenation restores mitochondrial respiratory function in hibernating myocardium (HM) cardiomyocytes. (a) Measurement of oxygen consumption rate (OCR) in cardiomyocytes following transwell co-culture with MSCs resulted in an overall increase in mitochondrial respiratory activity. (b) Basal and (c) maximal respiration were significantly increased following co-culture with MSCs ($p=0.0032$ and $p<0.0001$, respectively). (d) ATP production was significantly increased following MSC co-culture ($p<0.001$). Measurement of RNA expression of electron transport chain (ETC) complex 4 (e) and 5 (f) showed significant increases following co-culture with MSCs. (g) Measurement of nuclear-bound peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1 α) protein was significantly increased following MSC co-culture ($p<0.001$).

101.1 ± 2.6) and maximal (80.52 ± 8.21 vs 314.4 ± 9.4) respiration, as well as increased ATP production (24.56 ± 2.08 vs 83.2 ± 2.8) (Figure 2(a)-(d)). Quantitative real-time polymerase chain reaction of cardiomyocytes show decreased expression of electron transport chain proteins following exposure to hypoxia. Complex 4 is increased following reoxygenation (0.8228 ± 0.037 vs 1.114 ± 0.049) and treatment with MSC co-culture (0.8228 ± 0.037 vs 0.9821 ± 0.028) (Figure 2(e)). Complex 5—ATP synthase—is increased following reoxygenation (1.035 ± 0.022 vs 1.124 ± 0.031) and is further increased following co-culture with MSCs (1.035 ± 0.022 vs 1.235 ± 0.039) (Figure 2(f)). Complexes 1, 2, and 3 of the ETC were measured, but no significant changes were measured (data not shown). Nuclear-bound PGC1 α protein expression is increased following reoxygenation (0.013 ± 0.0024 vs 0.026 ± 0.0024) and is further increased following treatment with MSC co-culture (0.013 ± 0.0024 vs 0.0379 ± 0.0045) (Figure (g)).

Discussion

This study presents the first *in vitro* model of HM that exhibits maintained cellular viability and impaired function following chronic, low-grade hypoxia. We have also shown that the mitochondrial adaptations to HM in this model are not restored to normal function following reoxygenation alone (Figure 1(d)). This mimics the clinical experience in which CABG, the standard treatment of HM, may not fully restore cardiac function. Our previous work with a large animal model of HM has shown that hibernating cardiac tissue is characterized by decreased expression of ETC complexes, which is replicated in this model (Figure 2(e)-(f)), suggesting the mechanism of disease is related to mitochondrial function⁶⁻⁸.

We have previously identified the hallmark adaptations in HM tissue, which center around dysregulation of mitochondrial morphology, proteome, and function. Heart tissue has high energetic demand, making it critically dependent on effective mitochondrial function. The recovery of heart mitochondria in response to HM is dependent upon a dynamic process involving fission, fusion, and autophagy¹⁴⁻¹⁶. A critical balance between these variables is necessary to preserve mitochondrial energetics and contractile function in the ischemic heart¹⁷. We have previously demonstrated that inadequate mitochondrial bioenergetic capacity is present in HM pre- and post-CABG, as manifested by reduced expression of ETC proteins¹⁷. These observations suggest the process of mitochondrial dynamism is incomplete with CABG alone and that recovery of the mitochondrial proteome remains inadequate, despite evidence that cardiac tissue is no longer ischemic following CABG, as indicated by full restoration of regional blood flow in the HM region^{8,18}. Fusion protein (OPA-1, mitofusins 1 and 2) expression is necessary to re-establish normal mitochondrial energetics and function in the adult heart¹⁹. We have also demonstrated that PGC1 α , a driver of mitochondrial biogenesis, is significantly downregulated in HM and is not

restored following CABG¹⁷. Using transmission electron microscopy, we have observed in our HM model that mitochondria appear smaller and more variable in size despite CABG, which could be explained by the dysregulation of mitochondrial fusion, fission, and autophagy. This mitochondrial morphology does not normalize following CABG¹⁷. These observations identify the mitochondrial basis of HM, but clinical studies and animal models of HM are limited in their ability to allow the direct study of mitochondrial respiration. The development of this *in vitro* model aims to bridge this gap in appropriate research models of HM by creating a model that can allow for direct manipulation and measurement of mitochondrial respiration.

Stem cells provide a unique opportunity for addressing this underlying cause of cardiac dysfunction. Several recent clinical studies have advanced the notion that a cell-based therapeutic approach can either improve global function and/or reduce scar in patients with an ischemic cardiomyopathy. Studies in MI have shown that stem cell treatment results in increased PGC1 α signaling, which mediates mitochondrial biogenesis¹¹. The results of this study of *in vitro* HM indicate an increase in PGC1 α following reoxygenation and treatment with MSCs, which may indicate an increase in mitochondrial biogenesis in HM cells, resulting in increased mitochondrial function and respiration. By improving mitochondrial respiration, the ischemic heart is better equipped to respond to increased workload and can improve functional recovery following CABG.

There is a clinical need for an adjunct therapy to CABG that addresses the impairment of mitochondrial function seen in patients and animal models of this disease. Co-culture treatment of HM cardiomyocytes with MSCs shows improvement in both mitochondrial function and ATP production, both of which are critical for effectively functioning cardiac tissue. These data suggest a novel mechanism for the therapeutic potential of MSCs in heart disease that is independent of VEGF-mediated angiogenesis, which has previously been thought to be a primary mechanism of MSC therapy in ischemic tissue^{20,21}. As shown by our previous *in vivo* work in HM animals, as well as these *in vitro* findings, re-oxygenation alone is not enough to fully restore the function of HM tissue²². It follows that a therapeutic mechanism of angiogenesis alone would not be sufficient to explain the therapeutic benefit that is seen with MSC therapy in ischemic conditions. These results suggest that MSC therapy may act upon mitochondrial machinery by way of an increase in mitochondrial biogenesis to restore function. These results suggest that MSC therapy as an adjunct treatment to revascularization may address the current gap in treatment for HM patients. Identification of this therapeutic mechanism also suggests there may be a potential for MSCs to provide a benefit to impaired mitochondrial function in the absence of reoxygenation, which could be critical in instances of chronic ischemia that cannot be revascularized.

Ethical Approval

This study was approved by the Institutional Animal Care and Use Committee of the Minneapolis VA Medical Center.

Statement of Human and Animal Rights

All of the experimental procedures involving animals were conducted in accordance with the Institutional Animal Care and Use Committee guidelines of the Minneapolis VA Medical Center.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

Supplemental material for this article is available online.

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