An optimal non-viral gene transfer method for genetically modifying porcine bone marrow-derived endothelial progenitor cells for experimental therapeutics

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Abstract
No currently available treatment is able to generate new contractile tissue or significantly improve cardiac function after myocardial infarction (MI), a leading cause of morbidity and mortality worldwide. Although gene transfer-enhanced endothelial progenitor cells (GTE-EPCs) show effectiveness in MI treatment in small animal models, no clinical trials using GTE-EPCs have been documented. Before the introduction of GTE-EPCs into human trials, gene-transfer-mediated augmentation of EPC function in animal models that reflect the human MI scenario should be tested. In this regard, a porcine model is the best choice since pigs have cardiac size, hemodynamics and coronary anatomy similar to that of humans. To examine GTE-EPC therapeutic efficacy in pig MI models, an efficient method for gene transfer into pig EPCs is required, which however, has been poorly documented. Pig bone marrow mononuclear cells were isolated and cultured in EGM-2 medium to obtain bone marrow-derived EPCs (BM-EPCs) that were characterized by immunostaining and the tube formation assay. Gene transfer was optimized in 6-well plates using a GFP

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and a VEGF plasmid, and scaled up in T75 flasks. Gene transfer efficiency was determined by fluorescence microscopy and flow cytometry. VEGF levels were measured by ELISA. Cell proliferation was assayed by the CCK-8 kit. (1) BM-EPCs expressed VEGFR2 and eNOS but not CD45 protein, and formed tube structures on Matrigel; (2) several chemical compounds were explored with the highest transfection efficiency of 41.4% ± 5.8% achieved using Lipofectamine 3000; (3) the VEGF level in culture medium after VEGF transfection was 378 ± 48 ng/10⁶ cells; and (4) BM-EPCs overexpressing VEGF had significantly enhanced proliferation than GFP-transfected EPCs. A simple, easy and cheap method that can be applied to produce a large number of genetically-modified BM-EPCs was established, which will facilitate the study of GTE-EPC therapeutic efficacy in pig MI model.

**Keywords**
Myocardial infarction, endothelial progenitor cells, gene transfer, plasmid vector, pig model

**Introduction**

ST-elevation myocardial infarction (STEMI) is a leading cause of morbidity and mortality worldwide and despite advances in pharmacotherapy, greater accessibility of primary percutaneous coronary intervention, and development of international clinical guidelines for management, mortality at 1 year is more than 7%. The extent of resultant scar following a myocardial infarction (MI) is an important predictor of mortality, and no currently available treatment is able to generate new contractile tissue or significantly improve cardiac function. Supported by preclinical studies, cell-based treatment has emerged as an attractive modality for mitigating/reversing the effects of an MI in the clinical setting. Although the results of clinical trials have shown that adult stem cell therapy is safe and potentially efficacious, the functional benefit is modest and possibly due to impaired cell function caused by concomitant disease.

Two decades ago, Campbell et al. showed that the jugular venous delivery of allogeneic smooth-muscle cells, genetically modified to overexpress human endothelial nitric oxide synthase (eNOS), effectively prevented monocrotaline-induced pulmonary hypertension in rats. Since this first report, the regenerative and repair potential of enhanced cell treatment following acute myocardial injury has been extensively studied in animal models, with a focus on the genetic modification of stem/progenitor cells. A clinical trial of enhanced cell therapy using endothelial progenitor cells (EPCs) genetically modified to overexpress eNOS for acute anterior wall STEMI has been proposed. A prerequisite prior to the initiation of a clinical trial of genetically manipulated stem/progenitor cells is a clear understanding of all safety and efficacy aspects obtained in a large animal model of MI that reflects the human MI scenario. In this regard, a porcine model of MI is an attractive choice as pigs have cardiac size, hemodynamics, and coronary anatomy similar to that of humans.

Isolation and culture of pig EPCs either from peripheral blood or bone marrow (BM-EPCs) have been described in the literature. Muscari et al. compared different culture conditions and revealed that, in the presence of fibronectin and
VEGF, BM-EPCs exhibited endothelial cell behavior (i.e. formation of tube structure on Matrigel and expression of endothelial markers). Currently, recombinant plasmid and viral particles are the most commonly used vehicles for the delivery of genetic material.\textsuperscript{16,17} Although viral vector transduction affords high gene transfer efficiency, the technology is limited by issues of immunogenicity, complex preparation procedures, low packaging capacity, and expense.\textsuperscript{17} In contrast, plasmid vectors are safe, and easier and significantly cheaper to produce, but are associated with relatively lower gene transfer efficiency.\textsuperscript{17} To facilitate the selection of the most powerful gene for EPC-based gene therapy in pig models of MI, we tested different chemical reagents to establish an easy, simple and efficient gene transfer method for pig BM-EPCs.

**Methods**

**Isolation and culture of pig BM-EPCs**

The animal protocol was approved by the Animal Care Committee of St. Michael’s Hospital, Unity Health Toronto, University of Toronto (approval number: ACC893), in accordance with the NIH Guide for the Care and Use of Laboratory Animals, 8th edition. Male juvenile Yorkshire pigs weighing 30-35 kg were used for bone marrow extraction. Animals were anesthetized with intramuscular injection of ketamine (20 mg/kg), xylazine (2 mg/kg) and atropine (0.04 mg/kg) followed by inhalation of 5% isoflurane. Subsequently, the pig was placed in a supine position, and from the aseptically prepared iliac crest 20 ml of bone marrow were drawn into K2EDTA blood collection tubes (Becton, Dickinson & Co., Oakville, Canada). The blood was filtered through a 100-μm nylon cell strainer (Becton, Dickinson & Co.) and subsequently mixed with phosphate buffered saline (PBS, PH 7.4) in a 6 to 1 bone marrow to PBS ratio. Bone marrow mononuclear cells (BMMCs) were isolated with the density gradient centrifugation method using the Ficoll-paque media (GE Healthcare Canada, Mississauga, Canada). A 3 to 7 Ficoll-paque media to bone marrow ratio was used for Ficoll density gradient centrifugation as follows: 9 ml of Ficoll-paque media was added into a 50 ml centrifuge tube, and 21 ml of bone marrow/PBS mix was gently layered onto the top of the media. After centrifugation at 400 g at room temperature for 35 min, BMMCs were collected, washed twice with PBS and suspended at a density of 1.0 × 10^6/ml in EGM-2 medium, that is, endothelial basal medium (Lonza, Basel, Switzerland) supplemented with 5% fetal bovine serum and growth factors (VEGF, basic fibroblast growth factor and insulin-like growth factor-1).\textsuperscript{13,15} Ten ml of BMMC suspension was then seeded into each T75 flasks coated with 10 μg/ml human fibronectin and cultured at 37°C under a moist atmosphere containing 5% CO₂. After 48 h, medium with suspended cells was removed and fresh medium added. Cells were maintained with the change of medium every other day, passaged if cells became fully confluent for maximally three times and used after 14 days as described elsewhere.\textsuperscript{15}
Characterization of pig BM-EPCs

Immunostaining was done to determine the expression of CD45, VEGFR2, and eNOS protein in pig BM-EPCs. Two hundred μl of cells at a density of $1 \times 10^5$ cells/ml in EGM-2 medium were seeded into each well of a 48-well plate coated with human fibronectin (BD Biosciences, Mississauga, Canada) and cells were cultured for 24 h. The second day the staining procedure was completed at room temperature as follows: cells were washed one time with PBS and fixed with 4% formaldehyde for 20 mins (for eNOS staining, cells were washed 3 times with PBS followed by permeabilization with 0.5% Triton X-100 in PBS for 20 mins). Subsequently, cells were washed three times with PBS and blocked with 3% BSA in PBS for 30 mins followed by incubation for 1 h with mouse anti-VEGFR2 (Biolegend, San Diego, CA, USA, 1:50 dilution), mouse anti-porcine CD45 (Novus Biologicals, Littleton, CO, USA, 1:50 dilution) or eNOS antibodies (Abcam, Toronto, Canada, 1:50 dilution). Isotypes were used as controls. Afterwards, cells were washed three times with PBS and incubated with Alexa Fluor 555 goat anti-mouse IgG secondary antibody (Thermo Fisher Scientific, Missisauga, Canada) at a dilution of 1:1000 for 1 h in the presence of 1:5000 diluted nuclear dye Sytox Green (Thermo Fisher Scientific). After three washes with PBS, stained cells were visualized under a fluorescence microscope. The proportion of VEGFR2 and eNOS positive cells was calculated for each isolation and values from at least four randomly chosen fields were averaged.

In vitro tube-structure formation on Matrigel (Thermo Fisher Scientific, Missisauga, Canada) was carried out to assess BM-EPC angiogenic capability according to a previously described protocol with modification. HUVECs (Lonza) were used as control. Briefly, 100 μL of Matrigel was added into each well of a 96-well plate, and incubated at 37°C for 30 min. Subsequently, $2 \times 10^4$ BM-EPCs or HUVECs in 100 μl EGM-2 medium were added into each well and cultured at 37°C for 4 h. The tube-structure was then visualized and recorded using an inverted microscope.

Optimization of transfection

Three chemical reagents, that is, Lipofectamine 3000 (Thermo Fisher Scientific), SuperFect (Qiagen) and PolyJet (SignaGen Laboratories, Rockville, MD, USA) were explored for gene transfer into pig BM-EPCs. Optimization of cell transfection was done in 6-well plates and the transfection efficiency was determined using the plasmid vector pEGFP-N1 (Clontech, Mount View, CA, USA) expressing the green fluorescent protein (GFP). BM-EPCs were detached with 0.025% trypsin/EDTA, washed one time with PBS and re-suspended at a density of $1.25 \times 10^5$/ml in EGM medium. Two milliliter of cell suspension was added into each well of the 6-well plate that were previously coated with fibronectin (10 μg/ml in PBS) at room temperature for 1 h and cells were cultured for 24 h. Approximately 70% confluence was reached the second day and transfection was performed. Transfection was optimized for each reagent using different ratios of DNA and transfection
The highest transfection efficiency and minimal cytotoxicity were achieved using the DNA/reagent ratio suggested by the manufacturer. Transfection was performed as follows: (1) for transfection using Lipofectamine 3000, 2.5 μl of Lipofectamine 3000 and 2.5 μl of P3000 reagent were diluted in 62.5 μl of Opti-MEM Reduced Serum Medium (Thermo Fisher Scientific), and 1.25 μg plasmid DNA was diluted in 62.5 μl of Opti-MEM Reduced Serum Medium. The reagent and DNA dilutions were mixed and incubated at room temperature for 5 min, and then added into each well of cells that were washed one time with PBS and replaced with 2 ml fresh EGM medium prior to the addition of DNA/reagent mix. After cells were cultured for 24 h the percentage of GFP positive cells against total cells, for each isolation, was calculated and averaged from at least five randomly chosen fields; (2) for transfection using SuperFect, 1 μg DNA was diluted in 100 μl of endothelial basal medium followed by addition of 10 μl of SuperFect. After incubation at RT for 10 min, the DNA/SuperFect mix was added into cells and transfection efficiency was analyzed as described with Lipofectamine 3000 transfection; and (3) for transfection using PolyJet, 1 μg DNA was diluted in 50 μl of endothelial basal medium, and 3.0 μl of PolyJet was diluted in 50 μl of endothelial basal medium. Subsequently, the DNA and PolyJet dilutions were mixed, incubated at room temperature for 10 min, and added to cells. The transfection efficiency was analyzed as described with Lipofectamine 3000 transfection. Cell viability was assessed by trypan blue staining for all transfections.

**Transfection scaling-up**

Scaling-up of transfection using Lipofectamine 3000 was performed using T75 flasks. In addition to pEGFP-N1, pVEGF₁₆₅ (a plasmid vector expressing human VEGF₁₆₅ constructed in our laboratory) was also employed in scale-up experiments. pVEGF₁₆₅ has been used in a clinical trial to treat patients with advanced coronary disease, and we showed that conditioned media from pVEGF₁₆₅-transfected COS-1 and HEK293 cells was able to induce HUVEC proliferation while condition medium from non-transfected cells was not. Pig BM-EPCs were detached with 0.025% trypsin/EDTA, washed one time with PBS and re-suspended at a density of 2.0 × 10⁵/ml in EGM medium. Ten milliliter of cell suspension was added into each T75 flask and cultured for 24 h. The second day transfection was performed as follows: 20 μl of Lipofectamine 3000 and 20 μl of P3000 reagent were diluted in 500 μl of Opti-MEM Reduced Serum Medium, and 10 μg plasmid DNA was diluted in 500 μl of Opti-MEM Reduced Serum Medium. The reagent and DNA dilutions were mixed and incubated at room temperature for 5 min, and then added into each T75 flask of cells that were washed one time with PBS and replaced with 10 ml fresh EGM medium prior to the addition of DNA/reagent mix. Transfection efficiency using pEGFP-N1 was determined by microscopy as described above. The percentage of GFP positive cells was also determined by flow cytometric analysis as follows, second day after transfection, cells were detached with 0.025% trypsin/EDTA, washed one time with PBS, and re-suspended in PBS.
at a density of $2 \times 10^6$/ml. Half milliliter of cells were then analyzed using the Calibur Flow Cytometer ((Becton, Dickinson & Co.). The VEGF$_{165}$ level in the medium of cells transfected with the VEGF plasmid was measured by ELISA as described below.

**ELISA**

Twenty-four hours after transfection, the culture medium from BM-EPCs transfected with pVEGF$_{165}$ was collected, centrifuged at 12,000 g for 15 min at 4°C to remove cell debris, and VEGF content in the medium was then measured by a human VEGF ELISA Kit from R&D Systems (Minneapolis, MN, USA) according to the manufacturer’s instructions. The VEGF level was calculated and expressed as ng/10$^6$ cells. Conditioned medium from non-transfected or pEGFP-N1 transfected cells as control was also assayed by ELISA.

**In vitro assessment of BM-EPC proliferation after pVEGF$_{165}$ transfection**

To examine the functional effect of VEGF plasmid transfection on BM-EPCs, we compared cell proliferation between BM-EPCs overexpressing VEGF and GFP. BM-EPCs grown in a 6-well plate were transfected with the VEGF or GFP plasmid as described above. The second day after transfection, cells were detached and seeded in a 96-well plate in triplicate (2000 cells/well in 100 µl endothelial basal medium containing 0.1% FBS). Cells were then maintained in a cell culture incubator for 24 h followed by one wash with pre-warmed (37°C) PBS buffer. Subsequently, 50 µl of endothelial basal medium containing 5 µl of Cell Counting Kit-8 reagent (Sigma-Aldrich Canada Co., Oakville, ON, Canada) was added into each well, and incubated at 37°C for 4 h. The OD$_{450}$ value which is positively correlated with viable cell numbers was measured using a Spectramax M5 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, USA).

**Statistical analysis**

Transfection efficiency and cell growth data were expressed as average ± standard deviation. The ANOVA with post-hoc Tukey test was performed using the GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA) to compare the transfection efficiency among all reagents. A $p$-value of <0.05 was considered statistically significant.

**Results**

We generated pig BM-EPCs by culturing BMMCs using a protocol described by Muscari et al.$^{15}$ Immunostaining was performed for the characterization of BM-EPCs and the immunostaining images are shown in Figure 1. BM-EPCs are shown to express VEGFR2 and eNOS but not CD45. No fluorescent signals were observed with isotype staining (data not shown). Approximately 67% and 80% of BM-EPCs
Figure 1. Characterization of pig BM-EPCs. Immunostaining showed that pig BM-EPCs expressed VEGFR2 and eNOS protein but not CD45 as indicated in the figure. The percentages of VEGFR2 and eNOS positive cells are 67.5 ± 6.5% (n = 3 isolations) and 79.2 ± 15.9% (n = 3 isolations), respectively (the rightmost graph).
expressed VEGFR2 and eNOS, respectively (Figure 1). These BM-EPCs were able to form the tube-like structures when cultured on Matrigel (Figure 2).

Optimization of transfection was done using the GFP plasmid and BM-EPCs grown in 6-well plates. As shown in Figure 3, Lipofectamine 3000, SuperFect and PolyJet gave rise to a transfection efficiencies of $33.1 \pm 3.6\%$, $9.1 \pm 1.4\%$ and $1.6 \pm 0.4\%$, respectively (results from three cell isolations, and cells expressing eGFP show green fluorescence). Lipofectamine 3000 has a significantly higher transfection efficiency than the other two reagents ($p < 0.05$). Scaling-up of transfection was then performed using Lipofectamine 3000 with BM-EPCs grown in T75 flasks, and the results showed that $30.0 \pm 4.6\%$ of cells were positive for GFP (Figure 4). Flow cytometric analysis of GFP positive cells using non-transfected cells as control revealed that the transfection efficiency was $41.4 \pm 5.8\%$ (Figure 5(a), data from three isolations). Cell viability in both optimization and scale-up experiments was $>98\%$ as assessed by trypan blue staining.

Using the VEGF plasmid pVEGF$_{165}$ for scaling-up transfection, we demonstrated that the VEGF level in conditioned media from pVEGF$_{165}$-transfected BM-EPCs was $378 \pm 48$ ng/10$^6$ cells ($n = 3$) while the VEGF level in conditioned media from non-transfected or GFP plasmid-transfected BM-EPCs was below the detection limit of the ELISA kit (Figure 5(b)). Additionally, no fluorescence was observed in pVEGF$_{165}$ transfected BM-EPCs, which can serve as controls for eGFP plasmid transfection (data not shown). Furthermore, whether VEGF overexpression would enhance BM-EPC growth was examined. As shown in Figure 6, pVEGF$_{165}$-transfected BM-EPCs had an OD$_{450}$ value of $0.28 \pm 0.03$, which is significantly greater than $0.16 \pm 0.01$ of GFP plasmid-transfected BM-EPCs ($p < 0.05, n = 3$).

**Discussion**

Gene transfer to enhance the regenerative capacity of stem/progenitor cells has been extensively investigated in animal models of cardiovascular disease. However,
the vast majority of these studies used small animals (mice or rats). As the cardiovascular anatomy and hemodynamics of small animals differs dramatically from humans, the translation of findings from small animals to humans is limited, particularly for studies of EPC-based gene therapy for MI. A pig model of myocardial infarction provides an ideal platform for the assessment of EPC-based gene therapy before the initiation of clinical trials.

Pig EPCs can be isolated from peripheral blood or bone marrow. Peripheral blood EPCs are poorly proliferative, and the yield from culture is generally inadequate to allow for in vivo assessment of their regenerative capacity. With regard to the capability of pig peripheral blood EPCs to form tube structures on Matrigel, controversial findings have been reported. Luo et al. showed that pig peripheral blood EPCs formed tube structures, while Muscari et al. revealed the opposite. BM-EPCs rapidly proliferate, are able to form tube-structures in vitro, and therefore are preferred for the assessment of EPC-based gene therapy. Culture of human mononuclear cells in endothelial medium can generate two distinct types of cells: myeloid angiogenic cells (MACs) and EPCs. MACs express hematopoietic specific markers such as CD45 while EPCs do not. There is no
consensus on the identity (cell surface markers) of pig bone marrow derived endothelial progenitor cells. Currently, pig BM-EPCs are characterized by measuring the expression of endothelial cell specific markers\textsuperscript{13–15}. We showed that BM-EPCs did not express CD45 protein, which is also described in a previous study\textsuperscript{21} confirming that the cells we isolated were not MACs. Pig EPCs express endothelial markers such as VEGFR2\textsuperscript{14,15} and we showed that pig BM-EPCs robustly expressed both VEGFR2 and eNOS. Using an in vitro angiogenic assay, we showed that BM-EPCs formed tube-structures on Matrigel, which is in agreement with the finding reported by Muscari et al.\textsuperscript{15} A previous in vivo study demonstrated that iron-fluorophore labeled pig BM-EPCs were able to engraft in a pig model of myocardial infarction.\textsuperscript{21} Using MRI and immunohistochemistry Graham et al.\textsuperscript{21} showed persistence of delivered cells in the myocardium over a 6-week period. Taken together, BM-EPCs express endothelial markers, possess the capability to form tube-structures, and are able to engraft in the myocardium after transplantation, and therefore could be employed for the investigation of EPC-based gene therapy in pig myocardial infarction models.

**Figure 4.** Transfection scaling-up. Transfection was done in T75 flasks using Lipofectamine 3000. Similar transfection efficiency was achieved as with transfection using 6-well plates.
So far, gene transfer of pig BM-EPCs with plasmid has been poorly documented. Ward et al.\textsuperscript{22} used electroporation for the introduction of eNOS plasmid into pig BM-EPCs, and showed the coronary transplantation of BM-EPCs increased left ventricular ejection fraction in a porcine model of MI, but that eNOS overexpression did not provide any additional benefit. Electroporation requires an expensive device, and each electroporation can accommodate only $1 \times 10^6$ cells, making it laborious to produce a large number of cells for therapeutic applications.\textsuperscript{22-24} Additionally, after electroporation EPCs need to recover by re-seeding and overnight culture.\textsuperscript{22} In the present study, Lipofectamine 3000 was shown to be an excellent reagent for gene transfer into pig BM-EPCs with an transfection efficacy over 40% as determined by flow cytometric analysis. These results demonstrated that a large number of genetically engineered pig BM-EPCs can be obtained by transfection with Lipofectamine 3000. We further used a plasmid expressing human VEGF for transfection of BM-EPCs in T75 flasks, and a high yield of VEGF protein in cell culture medium was achieved, indicating genetically modified BM-EPCs can release the therapeutic product. Moreover, the functional effect of VEGF-transfection on EPCs was evaluated in vitro, and the results showed that pVEGF\textsubscript{165}-transfected BM-EPCs had a significantly greater proliferative rate than pEGFP-N1-

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\caption{Flow cytometric analysis of GFP and ELISA measurement of VEGF. Cells in scaling-up transfection experiments were analyzed for GFP positivity, and shown in panel A is a representative flow histogram. Black curve is for non-transfected cells as control while the pink one is for transfected cells. The number ($41.4 \pm 5.8\%$, mean ± standard deviation) is the percentage of cells positive for GFP from three independent assays. Panel B shows the VEGF expression levels.}
\end{figure}
transfected EPCs. These data suggest that, once transplanted in vivo, EPCs overexpressing VEGF not only exert paracrine effects but also possess augmented proliferation capability to promote angiogenesis for tissue repair.

Plasmid vectors for gene transfer are attractive because of their excellent safety, low-cost, and ease in production and that they can be used in human trials. A limitation of gene transfer by most plasmid vectors is transient transgene expression. With regard to gene-based cell therapy for cardiovascular disease, the requirement of short-term or sustained gene expression depends on disease pathogenesis. Short-term expression may suffice to induce processes such as vasculogenesis to mitigate myocardial damage following an acute MI, whereas sustained expression may be required to improve left ventricular function in heart failure. Human interferon \( \beta \) gene contains a scaffold/matrix associated region (S/MAR) that can bind chromatin and is conserved across eukaryotic cells. Therefore plasmids that carry S/MAR, which mediates the association of the plasmid with chromosome scaffold of host cells, can safely maintain in an episomal manner resulting in persistent gene expression. Papapetrou et al. showed that human myelogenous leukemia progenitor K562 cells expressed eGFP for 16 weeks after transfection with an eGFP plasmid harboring S/MAR. By a single tail vein injection with 50\( \mu \)g of

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**Figure 6.** The functional effect of VEGF transfection on BM-EPCs. BM-EPCs were transfected with either pEGFP-N1 or pVEGF\(_{165}\) and cell growth in medium without addition of growth factors was measured by the Cell Count Kit-8. As shown in this figure, pVEGF\(_{165}\) transfection led to a significantly higher number of viable cells compared with pEGFP-N1 transfection.
plasmid containing S/MAR to express luciferase in mice, Argyros et al.\textsuperscript{27} found that luciferase was detectable in the mouse liver 6 months after plasmid delivery. Such plasmid vectors for sustained gene expression can therefore be used if long-term gene expression is required to achieve therapeutic goals. We did not perform sample size calculation, which is a limitation of this study.

**Conclusion**

In conclusion, we established a high-efficiency gene transfer method for pig BM-EPCs with robust cell viability. This method is cheap and simple, and can be applied to produce a large number of genetically-modified BM-EPCs, which will facilitate studies of therapeutic efficacy of gene-transfer enhanced EPCs in pig MI models.

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**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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**Animal welfare**

The present study followed international, national, and/or institutional guidelines for humane animal treatment and complied with relevant legislation. The animal protocol was approved by the Animal Care Committee of St. Michael’s Hospital, University of Toronto (approval number: ACC893), in accordance with the NIH Guide for the Care and Use of Laboratory Animals, 8th edition.

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**Data availability**

All data related to this work are presented in the manuscript. If details of raw data are required, they are available upon request is made to the corresponding author.
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