Chronic nicotine impairs the angiogenic capacity of human induced pluripotent stem cell-derived endothelial cells in a murine model of peripheral arterial disease

Alex H. P. Chan, PhD,[a,b,c] Caroline Hu, BS,[c] Gladys C. F. Chiang, BS,[c] Chisomaga Ekweume, BS,[b,c,d] *and* Ngan F. Huang, PhD,[a,b,c,e] *Stanford, Palo Alto, and Davis, CA*

ABSTRACT

Objective: Lifestyle choices such as tobacco and e-cigarette use are a risk factor for peripheral arterial disease (PAD) and may influence therapeutic outcomes. The effect of chronic nicotine exposure on the angiogenic capacity of human induced pluripotent stem cell-derived endothelial cells (iPSC-ECs) was assessed in a murine model of PAD.

Methods: Mice were exposed to nicotine or phosphate-buffered saline (PBS) for 28 days, followed by induction of limb ischemia and iPSC-EC transplantation. Cells were injected into the ischemic limb immediately after induction of hindlimb ischemia and again 7 days later. Limb perfusion was assessed by laser Doppler spectroscopy, and transplant cell survival was monitored for 14 days afterward using bioluminescence imaging, followed by histological analysis of angiogenesis.

Results: Transplant cell retention progressively decreased over time after implantation based on bioluminescence imaging, and there were no significant differences in cell survival between mice with chronic exposure to nicotine or PBS. However, compared with mice without nicotine exposure, mice with prior nicotine exposure had had an impaired therapeutic response to iPSC-EC therapy based on decreased vascular perfusion recovery. Mice with nicotine exposure, followed by cell transplantation, had significantly lower mean perfusion ratio after 14 days (0.47 ± 0.07) compared with mice undergoing cell transplantation without prior nicotine exposure (0.79 ± 0.11). This finding was further supported by histological analysis of capillary density, in which animals with prior nicotine exposure had a lower capillary density (45.9 ± 4.7 per mm^2) compared with mice without nicotine exposure (66.5 ± 8.1 per mm^2). Importantly, the ischemic limbs mice exposed to nicotine without cell therapy also showed significant impairment in perfusion recovery after 14 days, compared with mice that received PBS + iPSC-EC treatment. This result suggested that mice without chronic nicotine exposure could respond to iPSC-EC implantation into the ischemic limb by inducing perfusion recovery, whereas mice with chronic nicotine exposure did not respond to iPSC-EC therapy.

Conclusions: Together, these findings show that chronic nicotine exposure adversely affects the ability of iPSC-EC therapy to promote vascular perfusion recovery and angiogenesis in a murine PAD model. (JVS—Vascular Science 2023;4:100115.)

Clinical Relevance: Cell therapy is a promising approach to induce revascularization in ischemic tissues associated with PAD. However, the role of lifestyle choices in modulating the efficacy of cell therapy is largely unknown. Using a murine model of PAD, this work demonstrates that chronic nicotine exposure can impair the ability of cell therapy to exert a therapeutic benefit. These results have important implications in the design of treatment options for patients with PAD with a history of tobacco or e-cigarette use.

Keywords: Endothelial cell; Angiogenesis; Limb ischemia; Nicotine; Induced pluripotent stem cell; Peripheral arterial disease

From the Department of Cardiothoracic Surgery,[a] Stanford Cardiovascular Institute,[b] Stanford University, Stanford; the Center for Tissue Regeneration, Repair and Restoration, Veterans Affairs Palo Alto, Health Care System, Palo Alto[c]; the College of Agricultural and Environmental Sciences, University of California Davis, Davis[d]; and the Department of Chemical Engineering, Stanford University, Stanford.[e]

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Correspondence: Ngan F. Huang, PhD, Associate Professor, Department of Cardiothoracic Surgery, Stanford University, 300 Pasteur Dr, MC 5407, Stanford, CA 94305-5407 (e-mail: ngantina@stanford.edu).

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Peripheral arterial disease (PAD) affects >8 million patients in the United States.[1] It is characterized by obstructive occlusion to the extremity vasculature that induces limb ischemia. PAD can result in pain, gangrene, and even limb amputation.[2] Current PAD treatment options include surgical or endovascular interventions, such as peripheral artery bypass grafting, angioplasty, and drug-eluting stents. However, these treatment strategies have poor long-term clinical outcomes with ≤50% of patients experiencing restenosis within 1 year.[3] Biological approaches to restore blood perfusion in response to limb ischemia hold promise for the treatment of PAD. Clinical trials include the delivery of angiogenic growth factors or adult therapeutic cells (ie, mononuclear cells, mesenchymal stem cells) to enhance angiogenesis. However, these biological therapies showed only modest benefit in prospective, randomized clinical trials.[4,5]

An alternative source of therapeutic cells is induced pluripotent stem cells (iPSCs), which are somatic cells that have been reprogrammed into a pluripotent state by the activation of key transcriptional factors.[6,7] Human iPSCs have nearly unlimited self-renewal capacity and differentiation capacity into endothelial cells (ECs) that initiate angiogenesis.[8] We and others have demonstrated previously that the delivery of human iPSC-derived ECs (iPSC-ECs) could restore blood perfusion and angiogenesis into mouse models of PAD.[9-12] Despite the potential therapeutic benefit of iPSC-ECs in preclinical models with well-controlled diets and lifestyle choices, it remains unclear whether iPSC-EC therapy would remain effective in the setting of nonideal lifestyles.

Lifestyle choices such as tobacco use are a major risk factor for PAD. Tobacco use is one of the strongest risk factors for the initiation and progression of PAD.[13] However, the pathological effects of the many tobacco smoke elements remain unclear. Nicotine, the addictive component of tobacco and e-cigarette products, is known to impair angiogenesis under chronic exposure.[14] A comparison of adipose-derived mesenchymal stem cells from smokers compared with nonsmokers revealed that smoker-derived stem cells had significantly impaired angiogenesis compared with nonsmokers.[15] In particular, the smoker-derived mesenchymal stem cells had compromised vascular tube-forming capacity, along with decreased expression of angiogenic secretome markers. These studies suggest that chronic nicotine exposure adversely affects the number and angiogenic capacity of endogenous stem cells. However, the effects of chronic nicotine use on iPSC-EC efficacy for treatment of PAD is unknown.[16]

In this study, we determine the effects of chronic nicotine exposure on the therapeutic efficacy of iPSC-EC implantation in a murine model of PAD (Fig 1). Nicotine was administered to mice via a subcutaneously implanted osmotic pump for 28 days before hindlimb ischemia surgery was performed on these mice. The iPSC-ECs were delivered intramuscularly to stimulate angiogenesis and vascular perfusion recovery. Although iPSC-EC survival was not affected significantly by nicotine exposure, vascular perfusion recovery was impaired significantly in mice receiving cell therapy with prior nicotine exposure, and this finding was confirmed by histological analysis of capillary formation. Our results underscore the important role of lifestyle choices such as nicotine exposure in affecting the angiogenic capacity of therapeutic cell transplantation for treatment of PAD.

ARTICLE HIGHLIGHTS

- **Type of Research:** Experimental research
- **Key Findings:** In an experimental model of peripheral arterial disease, mice without chronic nicotine exposure could respond to induced pluripotent stem cell-derived endothelial cell implantation into the ischemic limb by inducing perfusion recovery, whereas mice with chronic nicotine exposure did not respond to induced pluripotent stem cell-derived endothelial cell therapy.
- **Take Home Message:** Chronic nicotine exposure affects the potential therapeutic benefit of proangiogenic cell therapy adversely in a murine experimental model of peripheral arterial disease.

METHODS

Generation and characterization of iPSC-ECs. The human iPSC line (HUF4) was reprogrammed from dermal fibroblasts, as described previously,[9] and expanded in Essential 8 medium (Lonza, Basel, Switzerland). In brief, iPSCs were dissociated and grown in suspension in the presence of vascular endothelial growth factor (VEGF) and bone morphogenetic protein-4 (50 ng/mL) for 4 days, followed by reattachment onto gelatin-coated plates for 10 days in the presence of VEGF (50 ng/mL). The iPSC-ECs were purified from the samples using endothelial phenotypic cell-surface marker CD31 by fluorescence activated cell sorting. The purified iPSC-ECs were genetically tagged with a lentiviral construct to express firefly luciferase for detection by bioluminescence imaging, as described previously.[9,17,18]

To confirm their endothelial identity, the iPSC-ECs were stained immunofluorescently for the endothelial phenotypic marker, CD31, by immunofluorescence staining.[19-21] In brief, the cells were fixed in 4% paraformaldehyde before permeabilization in 0.1% Triton-X100 and 0.1% bovine serum albumin blocking. The primary antibody consisted of a mouse anti-human CD31 (Dako, Glostrup, Denmark). After primary antibody incubation and rinsing in phosphate-buffered saline (PBS), the cells were incubated with a secondary antibody consisting of Alexa fluor 594-conjugated goat anti-mouse (ThermoFisher

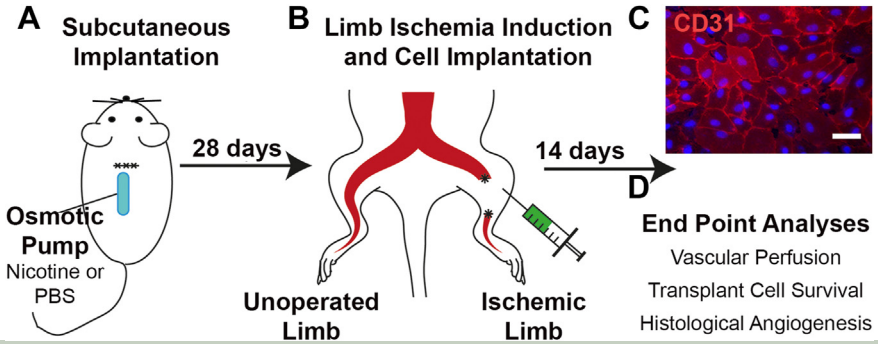

Fig 1. Schematic of study design. **(A)** Mice were exposed to systemic delivery of nicotine or phosphate-buffered saline (PBS) for 28 days through an osmotic pump implantation. **(B)** After 28 days of systemic pretreatment with nicotine or PBS, the mice underwent unilateral hindlimb ischemia, followed by injection of induced pluripotent stem cell-derived endothelial cell (iPSC-ECs) into the ischemic limb. **(C)** The human iPSC-ECs were characterized by the expression of endothelial phenotypic markers such as CD31. Additionally, the cells were transduced with a double fusion reporter construct to render the cells expressive for green fluorescence protein (GFP) and firefly luciferase. **(D)** For ≤14 days after cell transplantation, vascular perfusion recovery was monitored by laser Doppler spectroscopy, and the survival of transplanted iPSC-ECs was noninvasively assessed by bioluminescence imaging. After 14 days, the animals were humanely killed for histological assessment of angiogenesis. Scale bar, 50 μm.

Scientific, Waltham, MA). Total nuclei were visualized by a Hoechst 33342 nuclear dye (ThermoFisher Scientific).

Osmotic pump implant. All animal studies were approved by the Institutional Animal Care and Use Committee at the Veterans Affairs Palo Alto Health Care System. NOD SCID mice (male, 8-10 weeks old, 24-26 g) were anaesthetized by isoflurane. Osmotic pumps (2006, Azlet, Cupertino, CA) prepared with either nicotine (25 mg/kg/day; Sigma, St Louis, MO) or PBS only were implanted subcutaneously via a small incision in the dorsal side for 28 days before the induction of hindlimb ischemia (Fig 1, A). Osmotic pump delivery of nicotine continued until the animals were humanely killed at 2 weeks after cell delivery, for a total of 6 weeks of total nicotine exposure in the nicotine-treated mice.

Hindlimb ischemia. After 28 days of exposure to either nicotine or PBS pretreatment, the animals underwent unilateral hindlimb ischemia by ligation and excision of the common femoral artery from the inguinal ligament to the popliteal artery.[22,23] The mice were then treated with 1×10^6 bioluminescently labelled iPSC-ECs by intramuscular injection into the gastrocnemius muscle on day 0 and again on day 7 after hindlimb ischemia (Fig 1, B). Additional mice were randomized to receive nicotine pretreatment without cell implantation. On day 14 after induction of limb ischemia, the mice were humanely killed and the gastrocnemius muscle was explanted for histological analysis. Blood was collected and the serum was then extracted for validation of nicotine delivery by measuring cotinine levels using an enzyme-linked immunosorbent assay, based on the manufacturer's instructions (Calbiotech, El Cajon, CA).

Laser Doppler spectroscopy. Blood perfusion recovery of the hindlimbs were measured by laser Doppler spectroscopy at days 0, 3, 7, 10, and 14 after induction of ischemia (Fig 1, D). Mice were warmed with heated plate until a core body temperature of 37°C was measured with a rectal probe. Mice were then placed in a supine position for laser Doppler reading of the hind limbs. Blood perfusion was expressed as the mean perfusion ratio (perfusion of ischemic foot)/(perfusion of contralateral unoperated foot).[22,24]

Bioluminescence imaging. Implanted iPSC-ECs survival was tracked by bioluminescence imaging for ≤14 days (Fig 1, D). In the anesthetized state, the animals were injected intraperitoneally with D-luciferin (375 mg/kg body weight), and the mean radiance was monitored (IVIS Spectrum, Perkin Elmer, Waltham, MA) for ≥30 minutes until the bioluminescence signal reached a maximum.[25,26]

Histology and immunofluorescence staining. Explanted gastrocnemius muscle samples were embedded in optimal cutting temperature compound, frozen, and then cryosectioned transversely from proximal to distal at 10 μm. Slides were kept at −80°C until histological staining (Fig 1, D). Routine hematoxylin and eosin staining was performed to visualize the tissue morphology. For immunofluorescence staining, the tissue sections were thawed and fixed with 4% paraformaldehyde. The samples were permeabilized in 0.1% Triton-X100 and then blocked in 0.1% bovine serum albumin. Immunofluorescent staining was performed using an antibody targeting CD31 (BD Biosciences, Franklin Lake, NJ) for capillaries, smooth muscle α-actin antibody

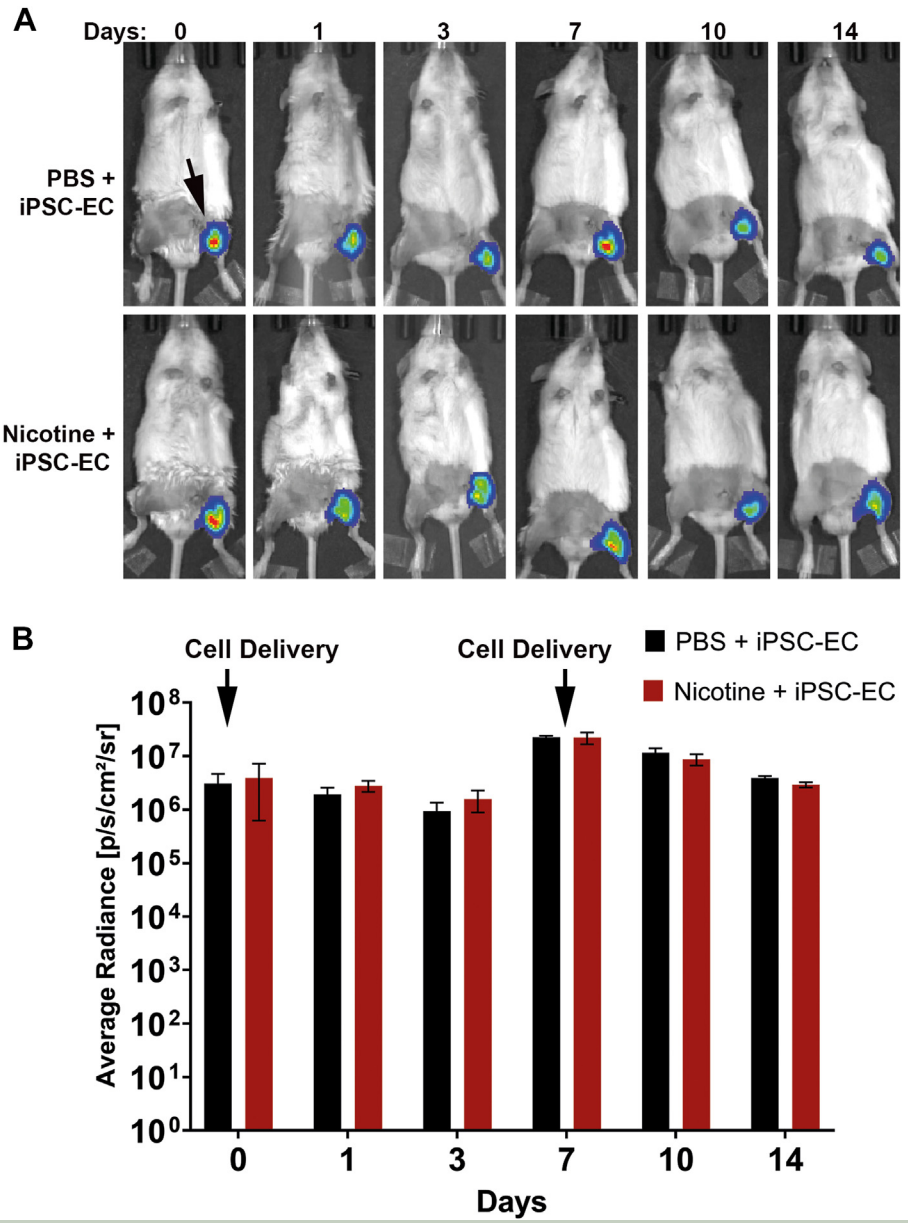

Fig 2. Noninvasive tracking of induced pluripotent stem cell-derived endothelial cell (*iPSC-EC*) transplant cell survival using bioluminescence imaging. In mice with prior exposure to nicotine or phosphate-buffered saline (*PBS*), the iPSC-ECs were implanted into the ischemic limb on day 0 and again on day 7 after induction of limb ischemia. **(A)** Representative images of cell survival over time. Arrow denotes ischemic limb. **(B)** Quantification of transplant cell survival. Data shown are mean ± standard error of mean (n = 8).

(Sigma) for arterioles, and CD206 (Abcam, Cambridge, UK) for M2 macrophages. After primary antibody incubation and rinsing in PBS, the cells were incubated with a Alexa fluor-594 or Alexa fluor-488-conjugated secondary antibodies (ThermoFisher Scientific). Total nuclei were visualized by a Hoechst 33342 nuclear dye (ThermoFisher Scientific). Images were captured using 10× objectives on an inverted microscope system (BZ-X710, Keyence, Osaka, Japan).

Image analysis. Quantification of capillary and arteriole densities were carried out according to our previous publication.[26] Three representative images were acquired for each tissue section, and the image files were named in a way that did not disclose the treatment group identity. Image analysis was performed in a blinded fashion, in which the individual who analyzed all of the histological samples had no knowledge of treatment group information. The number of CD31-expressing vessels were

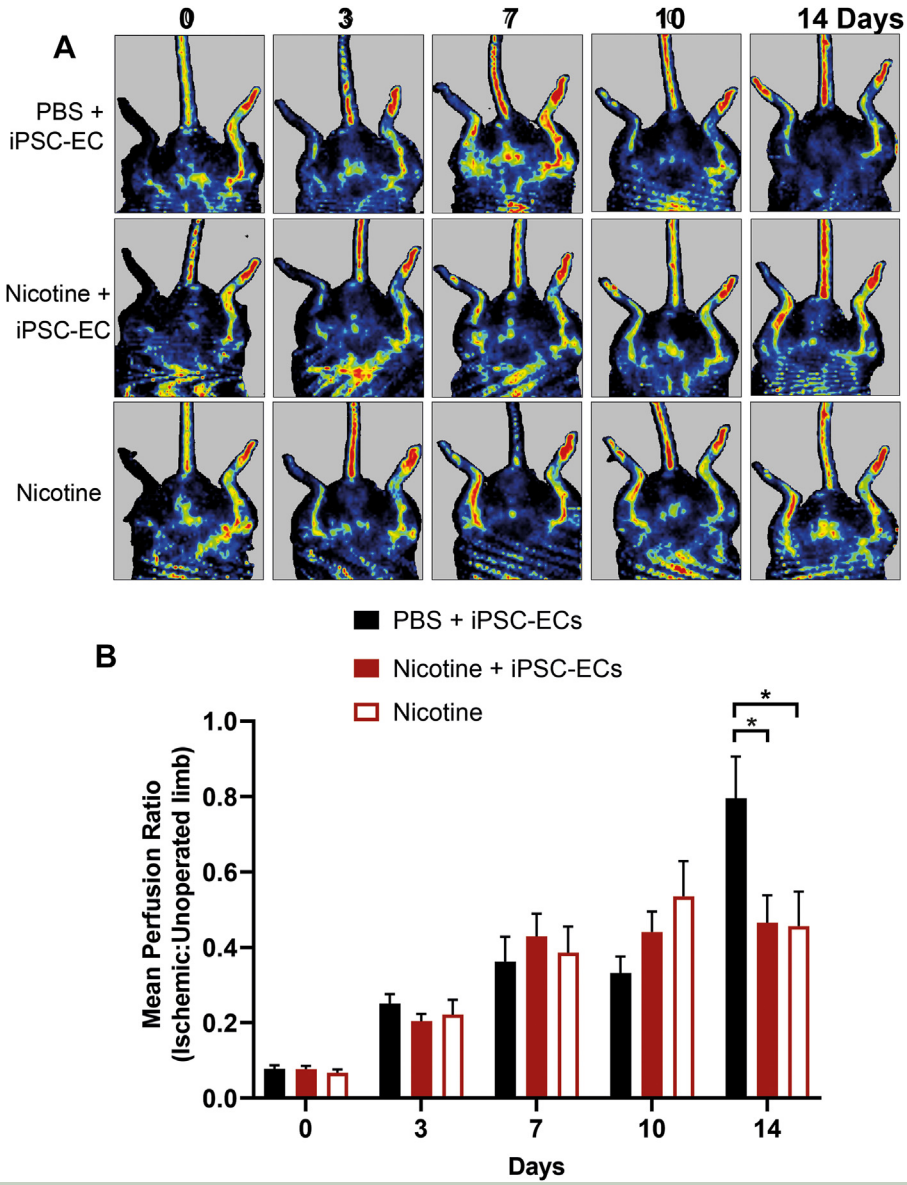

Fig 3. Laser Doppler spectroscopy analysis of limb perfusion recovery in mice with hindlimb ischemia after induced pluripotent stem cell-derived endothelial cell (*iPSC-EC*) transplantation. Before induction of limb ischemia, the mice were exposed to nicotine or phosphate-buffered saline (*PBS*) for 28 days. **(A)** Representative images of perfusion in the ischemic limb (denoted by arrow). **(B)** Quantification of relative mean perfusion ratio (ischemic/unoperated limb) for 14 days (*$P < .05$). Data shown are mean ± standard error of mean (PBS + iPSC-EC [n = 8]; nicotine + iPSC-EC [n = 10]; and nicotine [n = 6]).

counted and expressed as the capillary density (No. of CD31-expressing vessels/mm^2). Arteriole density was quantified in a similar manner, based on the number of SMA-expressing vessels/mm^2. The data were averaged among three tissue sections per animal. This method of quantifying capillary and arteriole density is well-established and accepted for the histological quantification of angiogenesis.[9,27-29] Additionally, to evaluate macrophage polarization in the ischemic limb tissue after iPSC-EC implantation, we immunofluorescently stained tissue sections for CD206 as an M2 macrophage phenotypic marker. CD206 staining was quantified using ImageJ thresholding, using a common threshold for all images and selecting for particles of positive staining that exceeded the size of cell nuclei and expressed as particle counts/mm^2. Area of interest for all analysis were taken from a cross-section of the gastrocnemius muscle where muscle regeneration occurred, as evidenced by the presence of centralized nuclei with in the myofibers.

Statistical analysis. All data are represented as mean ± standard error of the mean. The Shapiro-Wilk normality test was performed to verify normal distribution of the data. For comparison between the treatment groups in a

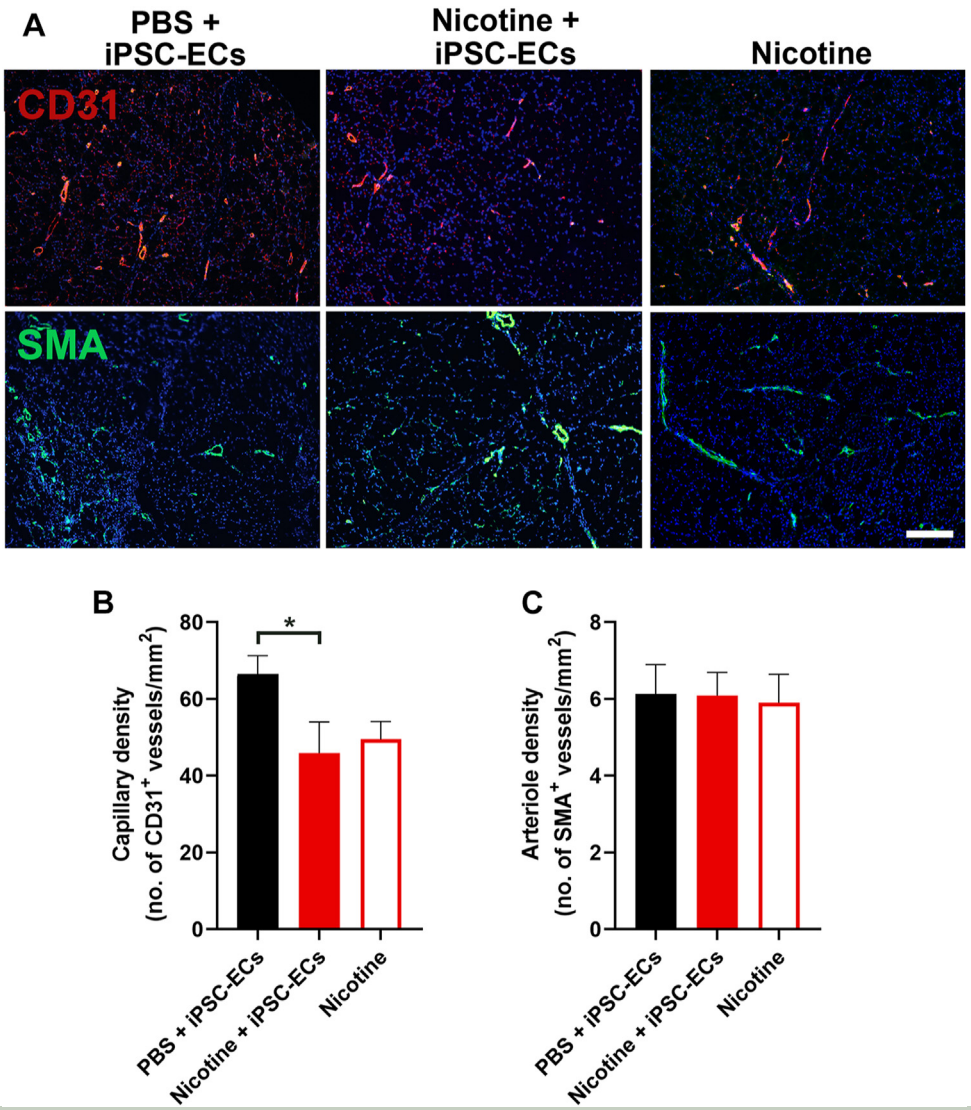

Fig 4. Histological analysis of revascularization by quantification of capillary and arteriole densities. **(A)** Representative images of CD31 and smooth muscle α-actin (*SMA*) staining. **(B)** Quantification of capillary density in the form of #/mm^2 (*$P = .034$). Data shown are mean ± standard error of mean (phosphate-buffered saline [*PBS*] + induced pluripotent stem cell-derived endothelial cell [*iPSC-EC*] [n = 11]; nicotine + iPSC-EC [n = 8]; and nicotine [n = 8]). **(C)** Quantification of arteriole density (#/mm^2) in PBS + iPSC-EC (n = 11); nicotine + iPSC-EC (n = 10); and nicotine (n = 7) groups. Scale bar, 200 μm.

murine model of PAD, an analysis of variance with Dunnett's multiple comparisons test. All statistical analyses were performed using GraphPad Prism statistical software. Values were considered to be statistically significant when the *P* value was <.05.

RESULTS

Transplant iPSC-EC survival. To examine the efficacy of cell therapy for treatment of PAD in individuals with chronic exposure to nicotine, we developed an experimental model in which mice received systemic nicotine delivery for 28 days before the induction of limb ischemia and then implantation of iPSC-ECs. The iPSC-ECs expressed the endothelial phenotypic marker, CD31 (Fig 1, C), as were well-characterized to be of endothelial identity from on our previous publications based on gene, protein, and functional analyses.[30-33] Furthermore, nicotine exposure was confirmed by serum cotinine levels to be 195.5 ± 23.0 ng/mL, which is consistent with reported serum levels to approximate more than one pack of cigarettes per week.[34]

To track the viable cells after implantation into the ischemic hindlimb, the cells were tagged with luciferase. Because systemically delivered D-luciferin substrate binds only to luciferase enzyme within viable transplanted cells, bioluminescence imaging could be used to quantify relative cell numbers over time as a measure of cell survival. In both PBS- and nicotine-treated mice, a

strong bioluminescence signal was detected in the hindlimb muscle on day 0 of cell implantation (Fig 2, *A*). Over the first 7 days, transplant cell survival gradually decreased , and so another dose of cells was delivered on day 7 to the ischemic hindlimb. Consistent with a second dose of cell therapy, the bioluminescence signal increased on day 7, followed by another gradual decrease in cell survival until day 14. The gradual decrease in cell survival was observed without significant differences between the groups at all time points (Fig 2, B; n = 8). These results suggest that nicotine exposure did not affect the viability of iPSC-ECs in ischemic limb significantly.

Perfusion recovery. Blood perfusion recovery of the ischemic hindlimb was measured using laser Doppler spectroscopy (PIM3, Perimed, Stockholm, Sweden) and quantified as mean perfusion ratio (ischemic/unoperated) (Fig 3, A). Importantly, on day 14 after induction of hindlimb ischemia, the mean perfusion ratio in the nicotine + iPSC-EC treatment group (0.47 ± 0.07; n = 10) was significantly lower ($P = .026$), compared with the PBS + iPSC-EC group (0.79 ± 0.11; n = 8), reflecting a decrease in perfusion levels by approximately one-half (Fig 3, B). Interestingly, the additional reference group receiving nicotine exposure without iPSC-EC implantation showed a mean perfusion ratio of 0.46 ± 0.09 (n = 6), which was also statistically significantly lower ($P = .046$) than the PBS + iPSC-EC group (0.79 ± 0.11; n = 8). These findings suggest that iPSC-EC treatment in a murine PAD model with chronic nicotine exposure may not be effective for augmenting vascular perfusion. Together, these findings demonstrate that mice without nicotine exposure could respond to iPSC-EC implantation into the ischemic limb by inducing perfusion recovery, whereas mice with nicotine exposure did not respond to iPSC-EC therapy.

Revascularization. To further corroborate the quantitative assessment on vascular perfusion recovery, we performed an end point histological assessment of the ischemic gastrocnemius muscle for revascularization. Capillary density was quantified using the endothelial marker, CD31 (Fig 4, A). Consistent with our laser Doppler analysis, the nicotine + iPSC-EC treatment group had a significantly lower capillary density (45.9 ± 4.7 per mm^2; n = 8) compared with the PBS + iPSC-EC group (66.5 ± 8.1 per mm^2, respectively; $P = .034$; n = 11) (Fig 4, B). The animals exposed to nicotine without cell therapy (45.5 ± 4.6 per mm^2; n = 8) showed nonsignificant trends in a decrease in capillary density, compared with the PBS + iPSC-EC group ($P = .086$). Arteriole density, which reflects more mature vessels, was examined by immunofluorescence staining of SMA (Fig 4, A). No significant differences were observed between the groups based on arteriole density (Fig 4, C). These results demonstrate that nicotine pretreatment before iPSC-EC transplantation modulated capillary formation, but not arteriole formation.

Because nicotine is known to modulate the inflammatory environment of the ischemic limb, we further assessed whether iPSC-EC treatment can sensitize macrophages toward an anti-inflammatory M2 phenotype. We performed immunofluorescence staining of CD206 and quantification of tissue sections as a marker of M2 macrophages. Our results show nonsignificant trends in higher M2 macrophage density in mice treated with iPSC-ECs, compared with nicotine alone (Supplementary Fig). However, there was no significant effect in macrophage polarization between the nicotine + iPSC-EC group compared with the PBS + iPSC-EC group.

DISCUSSION

The salient findings from this work are that (1) nicotine exposure did not affect the survival of intramuscular injected iPSC-ECs, compared with PBS exposure (Fig 2); (2) mice without nicotine exposure were capable of responding to iPSC-EC therapy by inducing perfusion recovery, whereas mice with prior nicotine exposure did not respond to iPSC-EC therapy (Fig 3); (3) histological analysis of capillary density demonstrated a significant decrease in capillary density in the nicotine treatment (Fig 4); and (4) nicotine did not have a significant benefit on M2 macrophage polarization, based on CD206 expression (Supplementary Fig). These findings suggest iPSC-EC treatment in a murine PAD model with chronic nicotine exposure may not be effective for augmenting angiogenesis and vascular perfusion. This study further suggests that chronic nicotine exposure may limit the clinical translation of angiogenic cell therapy for treatment of PAD.

Our studies suggest that chronic nicotine exposure affects the ability of the ischemic limb to respond to iPSC-EC therapy by restoring vascular perfusion. This effect was notable at 2 weeks after cell delivery. Nicotine could act either by decreasing baseline angiogenic capacity or by acting directly on transplanted cells. Nicotine interacts with ECs through nicotinic acetylcholine receptors (nAChRs), whose activity impacts other angiogenic pathways such as VEGF and fibroblast growth factor, which work synergistically to promote angiogenesis under acute nicotine exposure.[35] Nicotine has well-characterized transient angiogenic effects in vitro and in vivo.[36-39] However, there is evidence that chronic nicotine exposure reverses the angiogenic benefit.[14] In the context of chronic nicotine exposure, previous work indicates that nAChR-mediated nitric oxide (NO) signaling pathways play an important role in pathophysiology.[14] Studies involving second-hand smoke also showed endothelial dysfunction and decreased NO production,[40]

although these effects have not been linked to nicotine specifically. This result was observed in umbilical vein ECs, where the decrease in angiogenic function was postulated to be related to NO production.[41] Consistent with these data, decreased NO production was observed in rats under nicotine exposure over 4 weeks.[42] Although these studies suggest a link between chronic nicotine exposure, NO signaling, and impaired angiogenesis, there remains limited knowledge of the molecular mechanisms underlying how chronic nicotine exposure and NO signaling impair the angiogenic function of transplanted iPSC-ECs.

Another potential mechanism by which nicotine negatively impacts iPSC-ECs is in the decrease in circulating endothelial progenitor cells (EPCs). Chronic nicotine exposure has been shown to decrease the number of circulating EPCs significantly.[43] Li et al[43] showed that the expression of nAChRs of EPCs had decreased with chronic nicotine exposure, which led to decreasing activation of cholinergic angiogenic pathways. The authors also observed decreased expression of telomerase that affects the viability of EPCs, leading to a lower contribution to angiogenic process.[43]

Traditional cigarettes produce thousands of chemicals upon combustion[44] and e-cigarettes and vaping also result in a number of harmful chemicals when vapourised.[45] In our study, only nicotine was examined to reflect that commonality across tobacco-related products, including nicotine replacement therapy, in aiding tobacco cessation. Studies have aimed to examine the effects of tobacco smoke in vitro by including the majority of the chemicals that are released in tobacco combustion, using methods such smoke aerosol capture and smoke condensate for incorporation with cell media. Previous work shows that smoke condensate affects endothelial function negatively by disrupting microvascular structure and vessel size,.[46] To promote smoking cessation, consumers can choose the level of nicotine in the e-cigarette fluid and gradually decrease the nicotine levels over time. However, e-cigarette fluids without nicotine have been shown to decrease the proliferation of iPSC-ECs.[47] Adding nicotine to these e-cigarette fluids did not have additional negative effects on proliferation, but instead increased reactive oxygen species and increased apoptosis relative to non-nicotine e-cigarette fluids.[47]

In vivo studies using mainstream smoke or vaporized electronic fluid directly on rodent models show similar effects to in vitro studies, whereby whole stream smoke significantly diminished angiogenic function of the animals.[15,48] Most of these methods require mice to be physically restrained in a chamber for smoke inhalation, which can be traumatic and not feasible for chronic exposure experiments.[49] Using an osmotic pump with known release rates allows for the consistent delivery of nicotine for extended periods of time. In our study, the mice were exposed to nicotine for a total of 42 days with a singular osmotic pump, corresponding to nicotine levels in more than one pack per week. Compared with other methods of nicotine delivery, osmotic pump delivery of nicotine was reliable, based on the consistent serum cotinine levels (195.5 $\pm$ 23.0 ng/mL).

This study has several limitations. One limitation is the use of male mice for the preclinical studies to limit the potential influence of hormones from female mice. The use of male mice precludes the ability to study the potential role of sex differences in the response to nicotine and stem cell therapy in the setting of PAD. Another limitation is the lack of a PBS-only negative control group, which precludes the ability to determine the effect of chronic nicotine exposure in the setting of hindlimb ischemia. Nevertheless, our previous work comparing the therapeutic benefit of iPSC-EC therapy with PBS treatment supports that iPSC-EC therapy significantly improves vascular perfusion and angiogenesis.[9] In addition, we recognize that macrophage involvement in nicotine-induced inflammatory response is complex; therefore, further research is needed to elucidate the role of macrophage polarization.

CONCLUSIONS

We demonstrated that mice with prior chronic exposure of nicotine had impaired response to the benefit of iPSC-ECs in promoting vascular perfusion recovery and angiogenesis, when compared with mice with exposure to PBS vehicle control. The degree of cell survival was not significantly different between mice with or without nicotine exposure. These studies suggest that tobacco and e-cigarette history should be considered when considering angiogenic cell therapy in patients with PAD.

AUTHOR CONTRIBUTIONS

Conception and design: AHPC, NFH
Analysis and interpretation: AHPC, NFH
Data collection: AHPC, CH, GCFC, CE
Writing the article: AHPC, NFH
Critical revision of the article: AHPC, CH, GCFC, CE, NFH
Final approval of the article: AHPC, CH, GCFC, CE, NFH
Statistical analysis: AHPC, NFH
Obtained funding: AHPC, NFH
Overall responsibility: NFH

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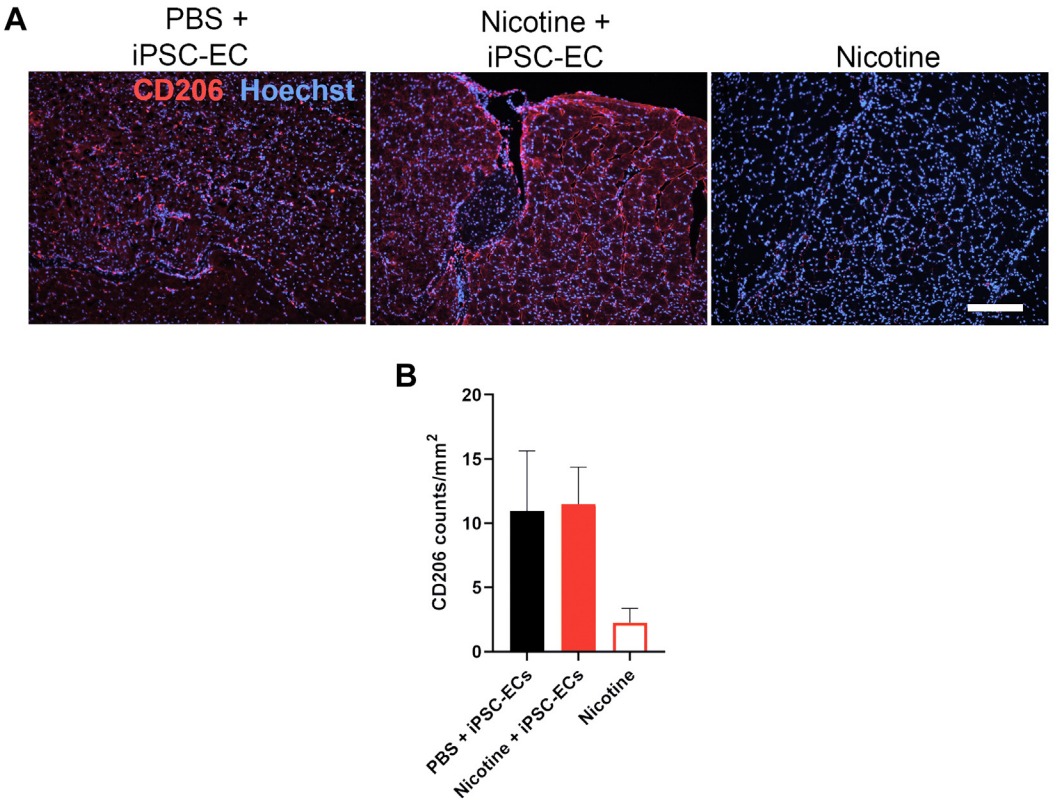

Supplementary Fig. Immunofluorescence staining of CD206 in ischemic limb tissue sections. **(A)** Representative images. **(B)** Quantification of CD206 density (counts/mm^2) in phosphate-buffered saline (*PBS*) + induced pluripotent stem cell-derived endothelial cell (*iPSC-ECs*) (n = 8), nicotine + iPSC-ECs (n = 9), and nicotine only (n = 6) groups. Data shown are mean ± standard error of mean. Scale bar, 200 μm.