Nucleofection induces transient eIF2α phosphorylation by GCN2 and PERK

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INTRODUCTION

Nucleofection is an advanced electroporation technique that varies electrical parameters and buffers to optimize delivery for specific cell types with high efficiency and reproducibility.¹ A major advantage of nucleofection is its versatility in transfecting a wide variety of primary dividing and non-dividing cell types.¹–³ Nucleofection can be used to deliver a variety of nucleic acids, including mRNA,⁴–⁶ small interfering RNA,⁴ microRNA,⁷,⁸ and DNA.⁹,¹⁰

An increasingly common use of nucleofection is the delivery of mRNA. Gene transfer based on mRNA is safe, because unlike DNA-based and viral vector approaches, mRNA-based gene transfer does not bear the risks of chromosomal integration.¹¹ Protein expression is rapid, beginning almost immediately upon mRNA reaching the cytoplasm. High transfection efficiency is obtained, in part because there is no requirement for mRNA to reach the nucleus.¹²–¹⁴ Unlike other gene-delivery strategies, no additional RNA transcripts are made following transfection, but mRNA degrades rapidly, and thus translation rates immediately following delivery are a key consideration for mRNA-based gene delivery applications.

A convergent response to cellular stress induced by a variety of insults is the phosphorylation of eukaryotic initiation factor 2 alpha (eIF2α), which results in a general decrease in translation initiation events and a global decrease in translation (for a review, see Holcik and Sonenberg¹⁵). There are four known eIF2α kinases in mammalian cells, each responding to different forms of cellular stress: RNA-dependent protein kinase (PKR), PKR-like endoplasmic reticulum (ER) kinase (PERK), general control non-derepressible-2 (GCN2), and heme-regulated inhibitor. Activation of PKR occurs principally with binding to structured RNAs,¹⁶ but can also occur through cellular protein-binding partners under stress conditions¹⁷ and is primarily characterized as an antiviral sensor, although it functions in multiple pathways.¹⁸ Heme-regulated inhibitor is active primarily in erythroid cells during heme deprivation.¹⁹ PERK is activated under conditions of ER stress as part of the unfolded protein response.²⁰ Lastly, GCN2 is stimulated by a variety of stresses, including amino-acid starvation,²¹ uncharged tRNAs,²² proteasome inhibition,²³ and ultraviolet irradiation.²⁴

Here, we show that nucleofection induces phosphorylation of eIF2α in the absence of a delivered nucleic acid, and GCN2 and PERK are the eIF2α kinases responsible for this phosphorylation. Furthermore, we show that translation is inhibited following nucleofection. The inhibition of translation resulting from this phosphorylation is potentially and clinically relevant, as it was found to occur in primary non-dividing human cells that are current targets of therapies involving nucleofection. The identification and understanding of non-specific effects of nucleofection are important for understanding the results obtained with its use. In addition, developing approaches to overcome nucleofection-induced eIF2α phosphorylation will enhance the use of nucleofection in clinical therapeutics.

RESULTS

Nucleofection induces eIF2α phosphorylation in WT MEF cells

The impact of nucleofection on translation was first studied in a mouse embryonic fibroblast (MEF) cell line derived from wild-type (WT) C57Bl/6 mice. Nucleofection conditions were optimized according to the manufacturer’s guidelines, and it was determined that program T-020 provided the best cell survival with high transfection efficiency. WT MEFs were then nucleofected without...
of the four mammalian eIF2 kinases, three: PKR, PERK, and GCN2, are widely distributed in all cell types, whereas heme-regulated kinase responsible for nucleofection-induced phosphorylation of eIF2α is reported to function primarily in erythroid cells. 19 of the mammalian eIF2α phosphorylation is a common feature of transfection, we treated the cells with Lipofectin, a commonly used cationic lipid, consisting of N-[1-(2,3-dioleyloxy)propyl]-n,n,n,trimethylammonium chloride, dioleoyl phosphatidylethanolamine and TransIT-mRNA (Mirus, Madison, WI, USA), a cationic polymer/lipid transfection reagent. Reagents were prepared and delivered according to the manufacturer's instructions, but without including any nucleic acid in the transfection mix. As a negative control, cells were mock treated by subjecting them to the same manipulation and buffers but without electric shock. Following nucleofection, eIF2α phosphorylation was assessed by western blotting using an antibody specific for eIF2α phosphorylated at serine 51. As shown in Figure 1, nucleofection induced phosphorylation of eIF2α fourfold over the baseline level present in mock-treated cells.

Lipid and polymer transfection reagents do not induce eIF2α phosphorylation in WT MEF cells

To determine whether eIF2α phosphorylation is a common feature of transfection, we treated the cells with Lipofectin, a commonly used cationic lipid, consisting of N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride, dioleoyl phosphatidylethanolamine and TransIT-mRNA (Mirus, Madison, WI, USA), a cationic polymer/lipid transfection reagent. Reagents were prepared and delivered according to the manufacturer's instructions, but without including any nucleic acid in the transfection mix. Neither lipid-based nor polymer/lipid-based transfection protocols induced phosphorylation of eIF2α (Figure 2).

GCN2 knockout reduces nucleofection-induced eIF2α phosphorylation

Of the four mammalian eIF2α kinases, three: PKR, PERK, and GCN2, are widely distributed in all cell types, whereas heme-regulated inhibitor is reported to function primarily in erythroid cells. 19 Therefore, to identify the eIF2α kinase responsible for nucleofection-induced eIF2α phosphorylation, we utilized MEF cell lines created from mice deficient in PKR, PERK, and GCN2. As was done with WT MEFs, cells were nucleofected and eIF2α phosphorylation was assessed by western blotting. Nucleofection of PKR−/− MEF cells resulted in eIF2α phosphorylation comparable to that induced in WT MEFs (Figures 3a and b), as did nucleofection of PERK−/− MEF cells (Figures 3c and d). Nucleofection also induced eIF2α phosphorylation in GCN2−/− MEF cells, but the peak level of phosphorylation was reduced (Figures 3e and f).

GCN2 phosphorylation is induced by nucleofection

A partial but incomplete reduction in eIF2α phosphorylation was observed only in GCN2−/− MEF cells; therefore, to confirm the role of GCN2, we examined GCN2 activation in WT cells following nucleofection. Activation of GCN2 was evaluated by assessing the level of phosphorylated GCN2 by western blotting. Nucleofection-induced phosphorylation of GCN2 was not observed in mock-treated cells, suggesting at least a partial role for GCN2 in nucleofection-induced phosphorylation of eIF2α (Figure 4).

Nucleofection reduces translation in a GCN2- and PERK-dependent manner

The removal of a single eIF2α kinase did not fully prevent nucleofection-induced eIF2α phosphorylation, suggesting the possibility that multiple kinases are involved. Therefore, we assessed phosphorylation of eIF2α following nucleofection of MEF cells derived from a dual-knockout GCN2−/−/PERK−/− mouse on a C57Bl/6 background. No eIF2α phosphorylation was observed in nucleofected GCN2−/−/PERK−/− MEF cells (Figure 5). An apparent lack of any baseline eIF2α phosphorylation that was seen in WT or single knockout MEFs was observed in these cells. The dual-knockout cells responded to polyinosinic:polycytidylic acid, demonstrating functional PKR and the ability of their eIF2α to be phosphorylated (Figure 5). These data demonstrated that both GCN2 and PERK were required for nucleofection-induced phosphorylation of eIF2α.

Nucleofection induces eIF2α phosphorylation in human dendritic cells

Nucleofection is increasingly used to deliver mRNA to human dendritic cells and T cells and has entered clinical trials. Therefore, we assessed the influence of nucleofection on eIF2α phosphorylation in primary human monocyte-derived dendritic cells (hMDDCs). As seen in MEF cell lines, nucleofection induced phosphorylation of eIF2α in hMDDCs (Figure 6). Furthermore, phosphorylation of GCN2 was also induced in hMDDCs following nucleofection but not mock treatment (Figure 6).

Nucleofection reduces translation in a GCN2- and PERK-dependent manner

The functional relevance of nucleofection-induced eIF2α phosphorylation was assessed by measuring translation following

Figure 1. Nucleofection causes phosphorylation of eIF2α in wild-type cells. Wild-type MEF cells were nucleofected or mock-treated and lysed at the indicated times. (a) Phosphorylation of eIF2α was assessed by western blotting with an antibody specific for phosphorylated eIF2α (eIF2α-P), and then re-probed for total eIF2α. Representative data from one of three independent experiments are shown. (b) Quantitation of western blot band densities. Values were calculated as the ratio of phosphorylated to total eIF2α and normalized to the values obtained in mock-treated cells at 0.1 h post shock. Data displayed are mean ± s.e.m. of n = 3 experiments. Asterisks indicate P-value <0.05 compared with mock treatment.

Figure 2. Cationic polymer and lipid transfection reagents do not induce phosphorylation of eIF2α in wild-type cells. Wild-type MEF cells were treated with the indicated transfection reagent or mock-treated and lysed at the indicated time points. (a) Phosphorylation of eIF2α was assessed by western blotting. Representative data from one of two independent experiments are shown. (b) Quantitation of western blot band densities. Values were calculated as the ratio of phosphorylated to total eIF2α and normalized to the values obtained in mock-treated cells at 0.1 h post shock. Data displayed are mean ± s.e.m. of n = 2 experiments.
nucleofection. One day before nucleofection, WT and GCN2\(^{-/-}\)/PERK\(^{-/-}\) MEF cells were transfected with a firefly luciferase expression plasmid. Cells were nucleofected in the absence of a nucleic acid and translation was measured by quantitation of luciferase enzyme activity. In WT cells, translation was decreased following nucleofection corresponding to the timeframe of eIF2\(^{\alpha}\) phosphorylation. However, nucleofected GCN2\(^{-/-}\)/PERK\(^{-/-}\) MEFs had a significantly smaller decrease in translation post nucleofection (Figure 7a). Nucleofection, in general, leads to toxicity and cell death, which was apparent in both cell lines as a reduction in translation that continued at 24 h and beyond.

Translation of a transfected nucleic acid was compared after directly delivering luciferase-encoding mRNA by nucleofection to WT and GCN2\(^{-/-}\)/PERK\(^{-/-}\) MEF cells. To circumvent RNA-induced activation of PKR, the \textit{in vitro} transcribed mRNA contained pseudouridines instead of uridines\(^{25}\) and was purified by high-performance liquid chromatography.\(^{26}\) Translation of nucleofected mRNA was significantly higher in GCN2\(^{-/-}\)/PERK\(^{-/-}\) MEFs than in WT MEFs by 1 h and continuing through 24 h following nucleofection (Figure 7b). To control for the effect of cell line derivation and clonality, the WT and GCN2\(^{-/-}\)/PERK\(^{-/-}\) MEF cells were transfected with the same luciferase-encoding mRNA using TransIT-mRNA, which, we demonstrated, did not result in phosphorylation of eIF2\(^{\alpha}\) (Figure 2). The WT MEF cells had slightly higher levels of translation (0 - 10%) throughout the time course, demonstrating that the reduction in translation of delivered mRNA was due to nucleofection.

Figure 3. The absence of GCN2 reduces phosphorylation of eIF2\(^{\alpha}\) following nucleofection. MEF cells deficient in the eIF2\(^{\alpha}\) kinases, PKR (a and b), PERK (c and d) or GCN2 (e and f), were nucleofected or mock-treated and lysed at indicated times. Phosphorylation of eIF2\(^{\alpha}\) was assessed by western blotting (a, c and e). Representative data of 2 to 4 independent experiments is shown. For quantitation of western blot band densities, values were calculated as the ratio of phosphorylated to total eIF2\(^{\alpha}\) and normalized to the values obtained in mock-treated cells at 0.1 h post-shock (b, d and e). Data displayed are mean ± s.e.m. of \(n=2\) to 4 experiments. Asterisks indicate \(P\)-value < 0.05 compared with mock treatment. Double asterisks indicate \(P\)-value < 0.05 compared with nucleofected WT MEFs.

Figure 4. Nucleofection induces GCN2 phosphorylation in WT cells. WT MEF cells were nucleofected or mock-treated and then lysed at the indicated time points. (a) Phosphorylation of GCN2 was assessed by western blotting. Representative data of three independent experiments are shown. (b) Quantitation of western blot band densities. Values were normalized to the values obtained in mock-treated cells at 0.1 h post-shock. Data displayed are mean ± s.e.m. of \(n=3\) experiments. Asterisk indicates \(P\)-value < 0.05 compared with mock treatment.
DISCUSSION

Nucleofection-mediated delivery of nucleic acids to cells in vitro, ex vivo and in vivo is an established approach that is now being utilized in clinical trials for the development of therapeutics. The advantage of this technique is that the efficiency of transfection of non-dividing cells is greatly increased. We demonstrate that nucleofection induces phosphorylation of eIF2α. Using knockout cell lines, we identify that the eIF2α kinases GCN2 and PERK are responsible for nucleofection-induced phosphorylation. Furthermore, nucleofection induced phosphorylation of eIF2α in primary human dendritic cells, indicating that this effect is relevant to the primary cell types currently under investigation for clinical therapeutics. Global and nucleofected mRNA translation was inhibited in WT MEFs following nucleofection, which was mitigated by absence of GCN2 and PERK, confirming the functional impact of nucleofection-induced eIF2α phosphorylation.

We identify that the electrical shock component of nucleofection leads to the activation of GCN2 and PERK and subsequent phosphorylation of eIF2α. This effect of electrical shock has not been previously observed and could be a common feature of electroporation or may be a specific effect of nucleofection. Underhill et al. examined eIF2α phosphorylation following electroporation, but did not observe an increase unless DNA was also included in the electroporation. These studies utilized the transformed CHO cell line and the EasyJet Plus (Equibio, Kent, UK) electroporation device and only analyzed phosphorylation of eIF2α 24 h later, a time when we typically do not see nucleoporation-induced phosphorylation of eIF2α. Tesfay et al. studied PKR−/− MEFs electroporated by a Bio-Rad GenePulser (Bio-Rad, Hercules, CA, USA). Examination of the eIF2α western blots suggested an increase in the amount of phosphorylated eIF2α 2 h after electroporation, but quantitation is not provided to allow accurate determination.

In WT cells and hMDDCs, phosphorylation of GCN2 was induced by nucleofection. In single-knockout cell lines, the absence of GCN2 resulted in a pronounced but not complete reduction in nucleofection-induced eIF2α phosphorylation, while no effect was observed with the absence of PERK alone. Elimination of nucleofection-induced eIF2α phosphorylation required the absence of both GCN2 and PERK. The observation that GCN2 phosphorylation coincides with eIF2α phosphorylation at early time points by GCN2 and at later time points by PERK. This is reminiscent of eIF2α phosphorylation in response to ultraviolet-C light, which is mediated by GCN2 at 1 h and by PERK at 4 h following ultraviolet exposure.

GCN2 is activated by a range of stresses, including nutrient limitation, proteosome inhibition, oxidizing conditions, high salinity and ultraviolet irradiation. In all cases, it is thought that GCN2 activation requires the binding of uncharged tRNA (see Dever et al. and Hinnebusch). PERK is an ER-associated transmembrane protein that normally exists in an inactive form as a heterodimer with the chaperone BiP. ER stresses, such as excess misfolded protein, cause dissociation of BiP, allowing PERK homodimerization and activation. In contrast to WT cells, in GCN2−/− cells, we observed a low level of nucleofection-induced

**Figure 5.** Phosphorylation of eIF2α is mediated by both GCN2 and PERK following nucleofection. GCN2−/−/PERK−/− MEF cells were nucleofected without nucleic acid (Nucleofected), mock-treated or nucleofected with poly(I:C), and then lysed at the indicated times. Phosphorylation of eIF2α was assessed by western blotting. Representative data from one of three independent experiments are shown.

**Figure 6.** Nucleofection of primary human dendritic cells induces phosphorylation of eIF2α and GCN2. Primary human MDDCs were nucleofected or mock-treated and lysed at the indicated times. Phosphorylation of eIF2α and GCN2 was assessed by western blotting. For quantitation of western blot band densities, values were calculated as the ratio of phosphorylated eIF2α or GCN2 to total eIF2α and normalized to the values obtained in mock-treated cells at 0.2 h post shock.

**Figure 7.** Nucleofection reduces translation in a GCN2- and PERK-dependent manner. WT or GCN2−/−/PERK−/− MEF cells were nucleofected and then lysed at the indicated time. Luciferase enzymatic activity was measured as relative light units (RLU). Asterisks indicate P-value <0.05 and daggers indicate P-value <0.001 comparing GCN2−/−/PERK−/− with WT MEFs. Data are representative of three independent experiments. (a) Cells were transfected with pCMV-luciferase plasmid 24 h before nucleofection. Luciferase activity was normalized to RLU present in non-nucleofected cells (mock) at the same time point. Data displayed are mean ± s.e.m. of four replicate wells. (b) Luciferase mRNA was delivered by nucleofection. Data are mean ± s.e.m. of three replicate wells.
elf2α phosphorylation, suggesting that GCN2 is the major kinase responsible for phosphorylating elf2α, and that nucleofection has an immediate impact on the availability of charged tRNAs. The absence of nucleofection-induced elf2α phosphorylation in GCN2−/−/PERK−/− cells suggests that the phosphorylation of elf2α seen in GCN2−/− cells results from PERK activation and occurs later than GCN2 activation following nucleofection. Nucleofection may directly cause ER stress, leading to PERK activation. Alternatively, nucleofection-induced PERK activation may occur as a consequence of the absence of GCN2. In this scenario, GCN2 activation following nucleofection results in translational repression and thereby prevents secondary ER stress. However, in the absence of GCN2, unpressed translation then leads to ER stress and PERK activation. Thus, although the data suggest that both GCN2 and PERK mediate nucleofection-induced elf2α phosphorylation, we cannot rule out whether GCN2 is, in fact, the only kinase. Attempts to measure phosphorylation of PERK were unsuccessful.

Nucleofection-induced elf2α phosphorylation typically returned to baseline levels by 4–6 h following nucleofection, although in some cases it extended through 24 h (data not shown). Given this timeframe, transgene expression following delivery of mRNA is likely to be affected most dramatically, although expression following plasmid transfection would be affected as well. In addition to reducing translation of the transfected gene, elf2α phosphorylation has a general impact by repressing global translation in cells. This is of particular concern for nucleofection of primary cells, which are often more sensitive and where a minimal disturbance can be detrimental. Numerous therapeutic approaches using mRNA delivery are under exploration, including transfection of dendritic cells with mRNA encoding tumor antigens, delivery of mRNA encoding vaccine antigens, cancer immunotherapy through transfection of T cells with mRNA encoding chimeric antigen receptors, stem cell research and induced pluripotent stem cell generation. Importantly, clinical trials utilizing nucleofection delivery are ongoing and trials with related technologies that deliver electrical stimulation applied to related technologies that deliver electrical stimulation applied to immunotherapies, including GCN4 and ATF4, and this property is dependent on the 5′UTR of these transcripts. Producing in vitro transcribed mRNAs containing the GCN4 5′UTR might allow selective translation throughout the duration of elf2α phosphorylation following nucleofection. We demonstrate that nucleofection of cells stimulates phosphorylation of the translation initiation factor elf2α in immortalized cell lines and primary cells. This phosphorylation is mediated by the kinases GCN2 and PERK, and results in global and delivered mRNA translational repression. In general, the primary consequence of elf2α phosphorylation is inhibition of translation; thus, for nucleofection-based gene delivery, the immediate impact is reduced translation of the nucleofected nucleic acid. In addition to decreased translation, phosphorylation of elf2α also leads to secondary consequences, including inhibited translation of most cellular transcripts, enhanced translation of stress genes and increased levels of apoptosis. Therefore, in addition to translational repression, other effects involving elf2α phosphorylation need to be considered. The efficacy of treatments could be limited both by translational repression and by cell stress sequelae, and therefore, elf2α phosphorylation must be considered in the design of nucleofection-based delivery approaches.

MATERIALS AND METHODS

Cells and reagents

Immortalized MEFs were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2 mM L-glutamine (Life Technologies, Carlsbad, CA, USA), 100 μM penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA), 10% fetal calf serum (HyClone, Logan, UT, USA), MEM non-essential amino acids (Invitrogen, Carlsbad, CA, USA), 55 μM β-mercaptoethanol (Bio-Rad). hMDDCs were prepared as described2 in serum-free AIM-V media supplemented with 50 ng/ml recombinant human granulocyte macrophage-colony stimulating factor and 100 ng/ml recombinant human IL-4. Polyinosinic-polycytidylic acid was purchased from Sigma (St Louis, MO, USA) and used at a concentration of 5 μg per 100 μl nucleofection.

Nucleofection

MEF cells were nucleofected using program T-020 and nucleofector V kit (Lonza, Basel, Switzerland). hMDDCs were nucleofected using program U-002 and human dendritic cell nucleofector kit (Lonza). After 15-min recovery in RPMI, cells were plated in complete media (DMEM/10% FBS and RPMI/10% FBS for MEFs and hMDDCs, respectively) and incubated at 37 °C with 5% CO2. At the indicated time following nucleofection, cells were lysed in RIPA lysis buffer supplemented with protease inhibitor cocktail (Sigma) and Halt phosphatase inhibitor (Pierce, Rockford, IL, USA) for western blotting.

Lipid and polymer transfections

Cells were seeded into 48-well plates at a density of 10 × 103 cells per well 1 day before transfection. Cells were exposed to 50 μL DMEM containing lipid-based lipofectin (Invitrogen), 50 μL DMEM medium alone or 200 μL complete DMEM medium containing polymer/lipid-based TransIT-mRNA (Mirus, Madison, WI, USA) for 1 h, which was then replaced with complete medium and further cultured. At the indicated time following exposure to lipid and polymer transfection reagents, cells were lysed as described for nucleofected cells.

Western blotting

Equal mass of protein (10–30 μg) for each sample was separated by 12% SDS-PAGE, transferred to Hybond-P PVDF membranes (GE Amersham), and probed with antibodies for GCN2-pT899 (Epitomics, Danvers, MA, USA), followed by HRP-conjugated anti-rabbit antibody (GE Amersham), and Pico or Femto chemiluminescent substrates (Pierce). Membranes were stripped by agitating gently in a buffer of 2% SDS, 0.05% Tween 20, and probed with antibodies for GCN2-pT899 (Epitomics, Burlingame, CA, USA) and elf2α-PS1 (Cell Signaling Technology, Danvers, MA, USA), followed by HRP-conjugated anti-rabbit antibody (GE Amersham), and Pico or Femto chemiluminescent substrates (Pierce). Western blots were captured using the LAS1000 digital imaging system (FujiFilm, Valhalla, NY, USA) and densitometry was performed using MultiGauge v2.2 software (FujiFilm).
Detection of reporter proteins in nucleofected cells
For plasmid-based reporter expression, 1 day before nucleofection, WT and PERK−/−GCN2−/− MEFs were transfected with pCMV-luciferase plasmid using Fugene-6 as described by the manufacturer (Roche, Basel, Switzerland). For mRNA-based reporter expression, in vitro transcribed firefly luciferase mRNA containing cap1, pseudouridine modifications and a 101-nt poly-A tail was generated, as previously described.25,26 Briefly, Megascrit T7 RNA polymerase (Ambion, Austin, TX, USA) was used to transcribe luciferase mRNA from linearized plasmid pTEVlucA101, replacing uridine triphosphate with pseudouridine triphosphate (Trilink, San Diego, CA, USA) in the transcription mix. The mRNA was captured using the m7G capping kit with 2′-O-methyltransferase (CellScript, Madison, WI, USA) to obtain cap1. The mRNA was then purified by high performance liquid chromatography as described.26 Nucleofection of WT and PERK−/−GCN2−/− MEFs was performed with 2.0 × 105 cells and 5 μg of luciferase mRNA per 100 μl nucleofection. Cell number and viability were monitored and were equal between treatment conditions at each time point.

Cells were trypsinized (0.05%, Life Technologies), nucleofected, seeded onto plates coated with collagen (0.01 mg ml−1) (Invitrogen), and lysed at the indicated time points in 25 μl cell lysis buffer (Promega, Madison, WI, USA). Two-microliter aliquots were assayed with the luciferase assay method for gene transfer into primary cells.27 Underhill MF, Coley C, Birch JR, Findlay A, Kallmeier R, Proud CG. The histidyl-tRNA synthetase-related sequence in the lumen of the endoplasmic reticulum. J Biol Chem 2001; 276: 630 - - 639.

Statistical analysis
All data are reported as mean ± s.e.m. Statistical differences between treatment groups were calculated by the Student’s t-test using Microsoft Excel. For all statistical testing, a P-value < 0.05 was considered significant.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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