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Evaluation of commercially available chemical reagents and electroporation for insertion of nucleic acids into hard-to-transfect cells

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ABSTRACT. Insertion of nucleic acids into cells unlocks the possibility of modulating gene expression; however, some cells such as primary cells and those that grow in suspension are hard-to-transfect. New therapies for cancer and possible autoimmune diseases, such as those that involve chimeric antigen receptor T cells, rely on the insertion of plasmids into lymphocytes, which fit into the hard-to-transfect category. In such cases virus-based transduction is usually applied, but the carrier vector tends to be incorporated into the cell's own DNA in a stable manner, with unpredictable consequences. Thus, highly efficient non-viral gene/plasmid delivery is a sought-after technology. We evaluated several commercially available chemical transfection methods as well as electroporation in difficult-to-transfect cells, including a human lymphocyte cell-line (Jurkat) and fresh peripheral blood mononuclear cells (PBMCs), both grown in suspension. The cell-toxicity of the methods was also evaluated. Twenty-four hours after transfection of the plasmid pCMV-GFP, the proportion of GFP positive (GFP+) cells was evaluated by cytometry. The cationic polymer TurboFect yielded ~7.8% of GFP+ Jurkat cells on average, while the other reagents (Lipofectamine 3000, FuGENE HD and X-tremeGENE HP) presented <3% of GFP+

cells. In PBMCs, none of the chemical reagents yielded >3% transfected cells. Electroporation was more efficient, with ~45% of GFP+ in Jurkat and ~15.7% GFP+ in PBMCs. However, it proved to be highly toxic, with ~80% of the cells considered non-viable 24h after the procedure, while TurboFect showed little-to-no toxicity. In conclusion, it was found that despite its high toxicity electroporation was the only method with applicable transfection efficiency in PBMCs, while in Jurkat the reagent TurboFect can be applied with acceptable results. The strategy for insertion of nucleic acids needs to be fine-tuned for each target cell type and experimental condition.

Key words: Chemical transfection; Electroporation; Plasmids; PBMCs

INTRODUCTION

One way of manipulating gene expression is through the insertion of nucleic acids into the cells, such as circular plasmids, RNAs such as interference small RNA or messenger RNA, among many others (Van Tendeloo et al., 2001; Mazurov et al., 2023). However, this is a difficult process to achieve since cell membranes do not allow nucleic acids to pass easily to protect the cell's internal machinery. Several methods have been developed over the past 50 years for insertion of DNA into cells: a- the ones that harbor biological machineries, such as virus-based particles for packing content; b- physical methods such as electro shocks, ultrasound and quick-temperature changes (heat-shock), among others (Mehier-Humbert and Guy, 2005); and c- chemical-based methods, such as covering the material with lipid- or polymer-based reagents that have affinity for the cell's membrane (Al-Dosari and Gao, 2009; Mazurov et al., 2023; Zhu et al., 2023).

Viral-based methods of inserting DNA into cells are quite effective, as they take advantage of the special ability of viruses to infect cells. However this is not the ideal method and non-viral gene delivery is a sought-after technology (Niidome and Huang, 2002; Lavigne and Gorecki, 2006; Al-Dosari and Gao, 2009). The main reason is that the inserted vector tends to be incorporated into the cell's own DNA in a stable manner, in various genomic sites and with unpredictable consequences and that is why this method is called transduction. Since the virus particles can infect any cell, thus proving hazardous to people handling them and the environment, a higher level of biosafety is required at the facility, and therefore not every lab can execute such experiments (Penna et al., 2010).

Gene therapy is a reality in our days, with some therapies already getting approved for clinical use and many others under development (Nardi et al., 2002; Cantore et al., 2021), such as the CRISPR-based systems with a promise to even fix disease-causing congenital genetic mutations such as sickle cell disease (Zarghamian et al., 2022). The insertion of plasmids in mononuclear cells from the peripheral blood (PBMCs) has become highly relevant in recent years thanks to the advent of a new technology for the treatment of cancer and possible autoimmune diseases, the CAR-T system (Chimeric antigen receptor T-cells) (Labanieh and Mackall, 2023). The methodology basically consists of the extraction of T or NK lymphocytes from the peripheral blood of the sick patient, genetic modification to force expression of a chimeric antigenic receptor – the so-called CAR's – *in vitro* expansion, and reintroduction of the modified cells into the patient. The extracellular part of the CAR contains the variable region of an antibody targeted at surface proteins found in

the cells that the cytotoxic modified lymphocytes (CAR-T) are supposed to destroy. Genetic modification is done by transducing or transfecting a plasmid containing the CAR gene (Labanieh and Mackall, 2023).

Primary lymphocytes fit into the group of hard-to-transfect cells. Cell-lines that grow adhering to surfaces, in a monolayer, such as HEK293T, HeLa, HEp-2, etc., are considered easy to transfect with chemical reagents. Cells that grow in suspension or form a colony, like the stem-cells, are usually more difficult for insertion of external nucleic acids. The situation is aggravated if they are primary cells, such as lymphocytes, for CAR-T therapy. In such cases, besides the virus-based delivery systems, a widely used physical method is electroporation. It was developed in the 80's and it seems to yield much higher transient transfection efficiency than chemical based methods do, but if the goal is to generate cell-lines with stable expression, electroporation is not as efficient as virus-based methods, circa 1% (Neumann et al., 1982; Sugar and Neumann, 1984; Chu et al., 1987; Chang, 2004). A downside of electroporation is low cell viability due to stress in the membrane and other effects (Beebe et al., 2003; Potter, 2003). Thus, various chemical carriers based in polymers, lipids and nanoparticles have been developed over the past couple decades that are non-viral and non-physical, promising low toxicity and high efficiency even in hard-to-transfect cells (Niidome and Huang, 2002; Lavigne and Gorecki, 2006; Ragusa et al., 2007; Guo and Huang, 2012).

In this study, we evaluated several chemical-based transfection methods as well as electroporation for difficult-to-transfect cells: a human lymphocyte cell-line (Jurkat) and PBMCs, both grown in suspension. The cell-toxicity of the methods was also evaluated.

MATERIAL AND METHODS

Cell culture

Jurkat cells, kindly donated by Prof. Troy R. Torgerson (from the Department of Pediatrics, University of Washington School of Medicine, Seattle, USA) were cultured with RPMI 1640 medium (#31800-014, Gibco) supplemented with 10% bovine fetal serum (#10BioPlus-500, Nova Biotecnologia, Brasil), + 1% antibiotic-antimycotic (#A5955, Sigma) + 1% L-glutamine, and kept in an incubator with atmospheric control (5% of CO2) and temperature control (37°C).The cells underwent maintenance every 48 hours so that the confluence would be kept at about 1 million cells per mL of medium.

The HEK293T cell-line (ATCC #CRL-3216), kindly donated by Dr. Alessandra Dellavance (from the Immunology Division, Fleury Laboratory, Sao Paulo, Brazil), was cultured with DMEN medium (#31600-034, Gibco) supplemented with 10% bovine fetal serum + 1% antibiotic-antimycotic + 1% L-glutamine, also with atmospheric and temperature control (5% CO2 at 37°C). For maintenance, upon reaching >80% confluence, cells were resuspended with trypsin and ~20% of the cells were reseeded with fresh medium.

Peripheral Blood Mononuclear Cells (PBMCs)

PBMCs were isolated from fresh human blood by density gradient (#17-1440-03, Ficoll-Paque PLUS 1.077 g/mL, Cytiva) as previously described (Higdon et al., 2016). The

human blood was collected from healthy donors in heparinized tubes so as to avoid coagulation. The same day after isolation, PBMCs were submitted to the transfection protocols, and cultured afterwards, just for short periods of time, until analysis in the cytometer. PBMCs culture conditions were similar to those of the Jurkat cells with the same RPMI 1640 supplemented culture medium, without proliferation stimulus. The research was approved by the institutional Ethics Committee at the Federal University of Sao Paulo (Plataforma Brasil CAAE: 08170919.1.0000.5505).

Transfection protocols

The Plasmid

The pCMV3-eGFP plasmid (SinoBiological, China) was applied in all transfections. It has a size of 6.7Kb, a kanamycin (KanR) resistance gene for bacterial amplification and the eukaryotic promoter pCMV (cytomegalovirus), which transcribes the GFP gene (green fluorescent protein). The plasmid was amplified in competent bacteria (*E. coli* DH5α, #C2987, NEB, USA) and purified with the Kit Fast-n-Easy Plasmid Mini-Prep (#DPK1045, Cellco, Brasil). Quantity and quality of purified material were accessed in a nanodrop spectrophotometer (ND-1000, ThermoScientific), and plasmid concentration ≥200ng/µL was used for the transfections.

Reagent-based transfections

One day before transfection, cells were seeded with supplemented Opti-MEM I Reduced Serum Medium (#22600-043, Gibco) in 24-well plates. For Jurkat and PBMCs, one hundred to two hundred thousand cells were seeded per well in 300µL of Opti-MEM. For HEK293T, cells were seeded in order to reach ~50-70% confluence on the transfection day, also in 300µL of Opti-MEM.

Transfections were carried out with Lipofectamine 3000 (Lot: 2422203, Cat:100022050, Invitrogen) diluted in pure Opti-MEM, following the manufacturer's protocol. As recommended, different proportions of transfection reagent x DNA were tested and the one with better performance was used in the repeated experiments, for example, 0.5µg or 1µg of DNA with 1µL or 2µL of reagent. For negative control, only 2µL of reagent was applied.

Tests with TurboFect reagent (Lot: 01153046Cat: R0531, ThermoFisher Scientific) were carried out according to the manufacturer's protocol, diluted in pure Opti-MEM. Proportions of DNA x reagent tested were: 0.5µg or 1µg of DNA with 2µL of TurboFect reagent. For negative control, 2µL of reagent was applied.

Tests with FuGENE HD (Lot: 0000463368Cat: E231A, Promega) were carried out according to the manufacturer's protocol, diluted in pure Opti-MEM. Proportions of DNA x reagent followed the recommendation of 1:3 (1 of DNA and 3 of reagent): 1µg of DNA for 3µL of Fugene HD, 2µg of DNA for 6µL of Fugene HD, 4µg of DNA for 12µL of Fugene HD. For negative control, 3µL of reagent alone was applied.

Tests with XtremeGENE HP (Lot: 62440700,Cat: 06366244001, Sigma-Aldrich) were carried out according to the manufacturer's protocol, diluted in pure Opti-MEM. Proportions of DNA x XtremeGENE HP reagent were: 1µg of DNA for 1µL of reagent,

1µg of DNA for 2µL of reagent, 1µg of DNA for 4µL of reagent. For negative control, 2µL of reagent alone was applied.

In all conditions, after ~4 hours of the transfection procedure, 500µL per well of the corresponding supplemented culture medium was added and the cells were maintained until the analysis of GFP expression was completed in the cytometer.

Electroporation

Electroporation was applied to transfect the plasmid pCMV3-eGFP into the cells that were grown in suspension, Jurkat and PBMCs. The protocol was followed as previously described (Chicaybam et al., 2013) and the following buffers were tested: **1SM** (5mM KCl; 15mM MgCl2; 120mM Na2HPO4/NaH2PO4 pH 7.2; 25mM Sodium Succinate; 25mM Manitol); **2M** (5mM KCl, 15mM MgCl2; 15mM HEPES; 150mM Na2HPO4/NaH2PO4 pH7.2; 50mM Manitol).

In brief, one million cells were centrifuged and the supernatant was discarded. Pellet was resuspended in 100µL of electroporation buffer (1SM or 2M) together with 4µg of DNA per sample. The material was transferred to a 0.2cm cuvette (#Z706086-50EA, Sigma-Aldrich) and electroporated with the program X-001 in the Amaxa Nucleofector II equipment. After the electroshock, 500µL of supplemented RPMI 1640 was added, incubated for 10 minutes at room temperature and the cells were transferred to a 24-well plate until analysis of GFP expression in the cytometer.

The live/dead assay

To evaluate cell viability, we applied the LIVE/DEAD Fixable Far Red Dead Cell Stain Kit (#L10120, ThermoFisher Scientific) according to the manufacturer's protocol. One day after the transfection procedure, cells were stained with the live/dead reagent; the stained cells were washed three times with PBS and analyzed in the cytometer.

Cytometry

Twenty-four hours after transfection, cells were washed once with PBS and resuspended in 300µl of cytometry buffer (#554657, BD Biosciences), and analyzed on a flow cytometer (CytoFLEX, Beckman Couter). One hundred thousand valid events gated from FSC-SSC (size and complexity) were collected for each sample. Expression of GFP was analyzed with the blue laser (Ex: 488nm) and with green filter (Em: 525nm). Live/dead stained cells were analyzed in the red laser (Ex: 640nm) and APC filter (Em: 660nm).

Data analysis

Cytometry data collected was analyzed with CytExpert v2.3 or Kaluza Analysis v2.1 software, and 30k to 50k events were shown in the representative graphs, as indicated in the figure legends. The data were presented as mean plus error bars indicating Standard Deviation (S.D.) or Standard Error of the Mean (S.E.M.). The graphs and tables were prepared with the help of Excel or GraphPad Prism v7.0 software.

RESULTS

We first tried to transfect the plasmid into the cells by using the commercially available lipid-based or polymer-based reagents. Transfection of the pCMV3-eGFP plasmid into the Jurkat cells by using the chemical reagents methods yielded poor results for the reagents Lipofectamine 3000, FuGENE HD and X-tremeGENE HP, meaning an average of <3% of cells expressing GFP after 24h (Table 1). In these cells, TurboFect presented better performance, with ~7.8% of cells expressing GFP, on average (Figure 1A and Table 1). As detailed in the methods, different proportions of transfection reagents x DNA were tested and the one with better performance was used in the repeated experiments (Table 1). In PBMCs, all the reagent-based methods we tested presented poor performance, with <3% of cells expressing GFP (Figure 1B and Table 1). The "easy-to-transfect" cell-line that grew in adherence, HEK293T, was used as a positive control for the reagents-based transfection protocols, and all reagents showed good performance, with some, such as Lipofectamine 3000 and X-tremeGENE HP, yielding on average ~60% of cells expressing GFP (Figure 1C and Table 1).

Table 1. Proportion of cells expressing GFP after transfection.

Cells	Transfection protocol	(*n*)	% of GFP (±S.E.M.)
Jurkat	Lipofectamine 3000	16	1.78 (±0.22)
	TurboFect	10	**7.84** (±1.66)
	FuGENE HD	10	0.35 (±0.11)
	X-tremeGENE HP	2	2.67 (±0.22)
	Electroporation	5	**44.97** (±13.68)
PBMCs	Lipofectamine 3000	3	2.29 (±0.23)
	TurboFect	12	0.43 (±0.22)
	FuGENE HD	10	0.56 (±0.10)
	X-tremeGENE HP	0	-
	Electroporation	6	**15.76** (±2.95)
HEK293T	Lipofectamine 3000	20	66.70 (±2.34)
	TurboFect	14	49.31 (±7.11)
	FuGENE HD	4	35.47 (±8.28)
	X-tremeGENE HP	3	59.61 (±2.20)

(*n*) indicates the number of times the experiment was repeated.

A common method of physically inserting DNA into "hard-to-transfect" cells is by electric shocks, or electroporation (Potter, 2003). Thus, we applied this method to insert the pCMV3-eGFP plasmid into Jurkat and PBMCs. In the Jurkat cells, electroporation was quite effective, with the yield of ~45% of GFP-positive cells, on average. In PBMCs, electroporation was the only method that worked when compared with the chemical reagents, with the yield of >15% of GFP-positive cells, on average (Figure 1A-B and Table 1). Although different electroporation buffers could be used, as described previously (Chicaybam et al., 2013), we tested two formulations: 1SM and 2M (see methods), and both showed similar performance. Thus, they were used randomly in the experiments.

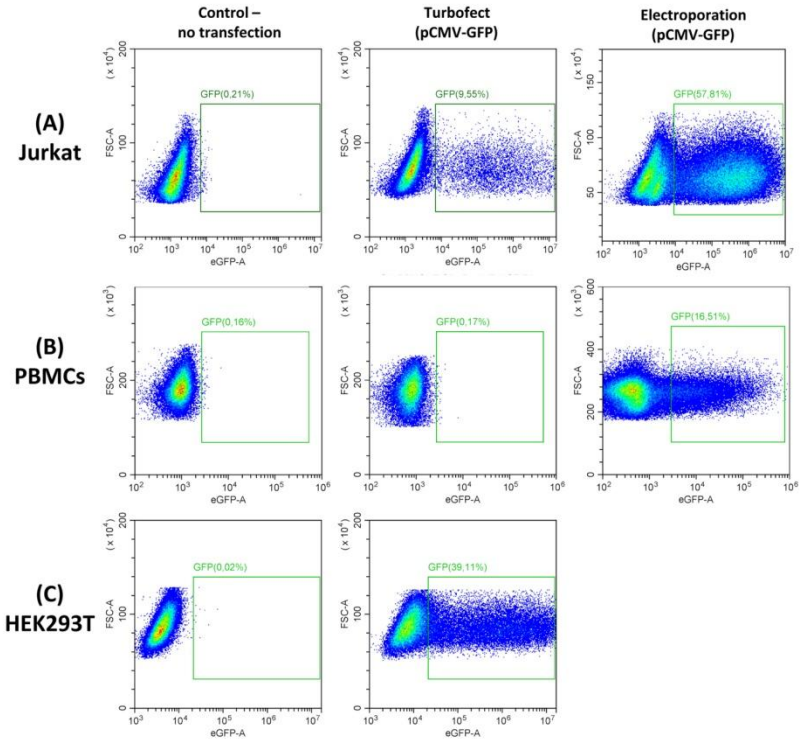

Figure 1. GFP expression after transfection. Cells were transfected by chemical methods (Table 1) or electroporation with the plasmid pCMV-GFP and analyzed 24h later. Representative cytometry graphs are shown with 50k events gated from FSCxSSC. (A) Proportion of GFP in Jurkat cells transfected with TurboFect or by electroporation. (B) Proportion of GFP in fresh PBMCs from a healthy donor, transfected by the given methods. (C) Positive control HEK293T cells, proportion of GFP after transfection with TurboFect.

Although electroporation is more effective in transfecting the cells, one downside of this method is the high cell toxicity due to changes in pH and salt concentrations, membrane instability, among others, as previously discussed elsewhere (Beebe et al., 2003; Potter, 2003). To evaluate cell viability after the transfection, we compared electroporation with TurboFect reagent, the chemical method with better transfection efficiency in the Jurkat cells. Based on the size and complexity (FSC x SSC) of the wild-type Jurkat, we first gated the viable cells (>80% of events) and named this gate the "OK" cells, as their size and complexity matched those of the wild-type Jurkat (Figure 2E). The TurboFect reagent, with or without the plasmid, showed little-to-no toxicity, meaning ~80% of the cells on average were within the gate for the cells with correct size and complexity corresponding to the viable cells (the "OK" cells) (Figure 2A-F). When submitted to electroporation, the proportion of viable cells was only ~10-20%, and >80% of the cells were considered non-viable "NV", as they presented smaller sizes (Figure 2C-F). The toxicity came from the electroporation procedure as cells submitted to the process alone, without plasmids, also presented similar low viability (Figure 2C).

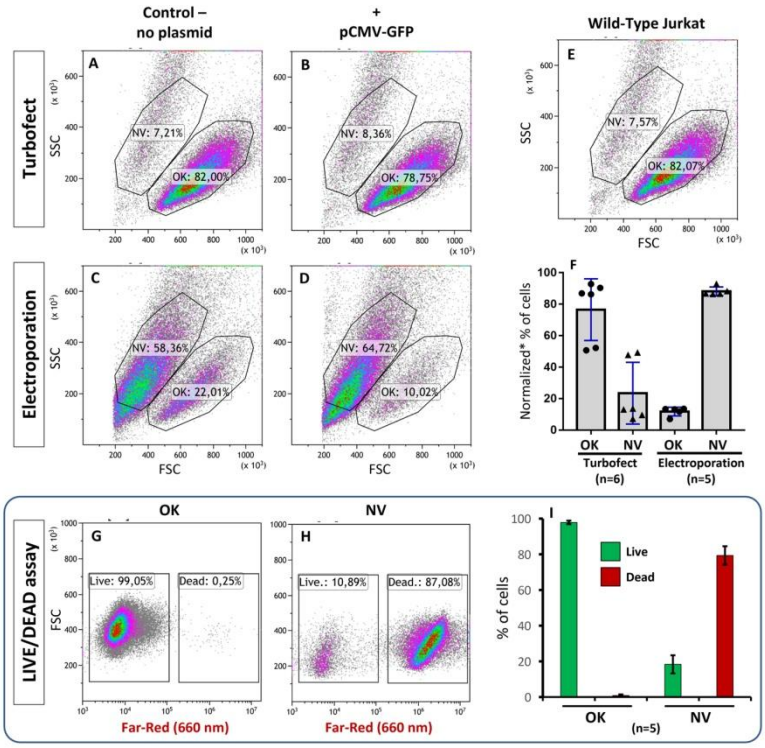

Figure 2. Cell toxicity by the transfection protocols. Proportion of cells with size and complexity corresponding to viable (OK) or non-viable (NV) Jurkat cells was evaluated 24h after TurboFect (A–B) or electroporation (C–D). (A–F) Viable (OK) and non-viable (NV) cells were first gated based on size and complexity (FSCxSSC). (F) For normalization *number of events within OK plus NV were considered as 100%, and the proportion within each gate was calculated. (G–I) A fluorescent reactive dye, which could penetrate and stain dead cells, was applied to Jurkat cells 24h after electroporation. (G) Live/dead cells gated from OK. (H) Live/dead cells gated from NV. (I) Proportion of dead cells in OK and NV. (F and I) Error bars = S.D.

To confirm that the gated "NV" cells were actually dead, we stained the cells with a reagent that could penetrate and stain only the dead cells (Live/Dead Assay). Indeed, ~80% of the cells from the NV gate were stained, meaning they were dead cells, while in the "OK" gate, ~99% of the cells were considered viable without any staining (Figure 2G-I), thus suggesting that cell viability was affected by the electroporation procedure.

DISCUSSION

The biggest experimental challenge currently for *in vivo* application of gene therapy is the delivery of gene-altering tools to the correct cells (Galarreta and Lujambio, 2017; Li et al., 2021; Tsai et al., 2022). The process also has not yet been completely mastered in the case of *in vitro* applications. Our goal was to evaluate if commercially available chemical reagents could be applied to insert plasmids into hard-to-transfect cells, including PBMCs. Unfortunately for use in PBMCs, none of the reagents we tested proved effective; though we managed to insert the plasmid into the PBMCs with electroporation, although this

method showed the downside of low cell viability after the procedure was completed, as extensively demonstrated elsewhere (Beebe et al., 2003; Potter, 2003; Chang, 2004).

In Jurkat cells, the recently marketed product, TurboFect, by TermoFisher yields satisfactory results although, depending on the experimental requirements, an ~8% proportion of cells expressing the desirable protein may not be good enough. TurboFect is a proprietary cationic polymer which forms complexes with the DNA and is readily endocytosed. We have also tried Lipofectamine 3000, a lipid-based reagent that has been on the market for over 20 years and has been successfully applied in thousands of studies to transfect all kinds of nucleic acids. Regarding the other reagents used in this study, FuGENE HD is a non-liposomal reagent and X-tremeGENE HP is a polymer-based multi-component reagent. All these reagents are marketed as being effective for hard-to-transfect cells. Our laboratory previously utilized the X-tremeGENE HP reagent to successfully transfect plasmids into insect cells (data not shown).

In conclusion, despite its high toxicity, electroporation is the only method with applicable transfection efficiency in PBMCs, while in Jurkat, the reagent TurboFect can be applied, as well as electroporation, with acceptable results. We confirm that delivery of nucleic acids into cells that grow in suspension is not an easy task, or at least not as easy as in HEK293T cells where most reagents seem to display great efficiency. The protocol will have to be fine-tuned for each experimental condition; for example, for cell-lines such as the Jurkat, chemical-reagents such as TurboFect may be a viable option. If the goal is to transfect PBMCs either for transient transfection or generation of stable cell-lines, the options we recommend would be electroporation or virus-based delivery, although electroporation has shown high toxicity in our experiments. In short, for successful gene and cell therapy, especially in clinical applications for treatment of cancer such as CAR-T, or to fix disease-causing genetic mutations with CRISPR/Cas-based technology, improved methods for gene delivery will have to be developed and made commercially available. To be specific, non-viral procedures based on improved safety and simplicity need to be developed; these should have high efficiency and low toxicity not only for *in vitro* but also for *ex vivo* and *in vivo* applications.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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