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Cardiomyocytes Permeabilization and Electrotransfection by Unipolar and Bipolar Asymmetric Electric Field Pulses

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Abstract

Short electric field pulses represent a novel potential approach for achieving uniform electroporation within tissue containing elongated cells oriented in various directions, such as electroporation-based cardiac ablation procedures. In this study, we investigated how electroporation with nanosecond pulses with respect to different pulse shapes (unipolar, bipolar, and asymmetric) influences cardiomyocyte permeabilization and gene transfer. For this purpose, rat cardiomyocytes (H9c2) were used. The efficacy of the pulsed electric field protocols was assessed by flow cytometry and electrogene transfer by fluorescent and holotomographic microscopy. The response of the cells was assessed by the metabolic activity (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT] assay), F-actin distribution in cells by confocal microscopy, and muscle atrophy F-box (MAFbx) marker. We show nano- and microsecond pulse protocols, which are not cytotoxic for cardiac muscle cells and can be efficiently used for gene electrotransfection. Asymmetric nanosecond pulsed electric fields were similarly efficient in plasmid delivery as microsecond and millisecond protocols. However, the millisecond protocol induced a higher MAFbx expression in H9c2 cells.

Keywords: cardiomyocytes, electroporation, asymmetric pulses, electro-gene-transfer

Introduction

Nanosecond pulsed electric fields (nsPEFs) have recently been widely used in various biological, medical, and food applications. It is known that nsPEFs can impact intracellular organelles and alternate biological mechanisms.^{1–3} Ultrashort electric pulses, depending on their number, frequency, and intensity, can stimulate either physiological or cell-destructive processes.^{4–6} Based on nsPEF's sensitizing properties of inner and outer membranes,^{7,8} most of the available studies focus mainly on the usability of nsPEFs in transmembrane transport of ions, drug molecules, nanocarriers, or nucleic acids.^{9,10} The most challenging seems to be gene electrotransfer (GET), as plasmids are relatively big molecules. Electroporation technology using milli- and microsecond pulses was specifically used in biological vector delivery.^{11,12} The nanosecond range is still fresh in this field

and requires further development. In this study, we aimed to utilize unipolar (UP) and bipolar (BP) asymmetric electric nanosecond pulses for plasmid DNA (pDNA) delivery to cardiomyocytes *in vitro*.

Material and Methods

Cell culture

Rat cardiac myoblast cell line (H9c2[2-1]) (CRL-1446, ATCC[®], mycoplasma free) was used in the study. CT26.WT (CRL-2638, ATCC[®], mycoplasma free) undifferentiated colon carcinoma cells were used as an additional electroporation susceptible control for the permeabilization study. H9c2 and CT26.WT cells were cultivated in Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich, Germany) supplemented with 4 mM L-glutamine (Sigma), 10% FBS heat-inactivated (Gibco[™], Life Technologies, Poland), and

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1% antibiotic–antimycotic solution (Merck Life, Poland) at 37°C in a 10% CO₂ atmosphere in a 5% humidified incubator (Binder, VWR).

Exposure of cells to electric pulses

For the pulsed electric field treatment, H9c2 and CT26.WT cells were trypsinized and centrifuged (5 min × 1500 rpm; Centrifuge 5430R, Eppendorf AG, Hamburg, Germany). Then, the cells (2×10^6 cells/mL) were resuspended in Sucrose-HEPES-Magnesium (SHM-HEPES)-based buffer used for electroporation, as described previously.^{13,14} Electroporation was performed in cuvettes with a 1 mm gap between electrodes (BTX, Syngen Biotech, Poland). BTX ECM 830 (Harvard Apparatus, Holliston, MA, USA) electric pulse generator was used to deliver European Standard Operating Procedures of Electrochemotherapy (ESOPE) ($1.3 \text{ kV/cm} \times 100 \mu\text{s} \times 8$, and 1 Hz) and the millisecond protocol ($0.6 \text{ kV/cm} \times 5 \text{ ms} \times 8$, and 1 Hz). A high-frequency BP electroporator¹⁵ was used for the generation of nanosecond pulse sequences (UP or BP). The summary of the applied protocols is shown in Figure 1. The nanosecond pulses were delivered in a burst of 100 pulses with a 500 kHz repetition frequency, and a total of four pulse sequences were used (SEQ 1–4), which are combinations of $7 \text{ kV/cm} \times 700 \text{ ns}$ and $12 \text{ kV/cm} \times 300 \text{ ns}$ pulses (Fig. 1). For permeabilization study, the effects of pulse amplitude for both the 300 and 700 ns pulses were studied in the 1–14 kV/cm range. Multiparametric permeabilization study was used to derive SEQ 1–4 (aiming for a high cell membrane permeabilization rate, which is crucial for electrotransfection).

Cell membrane permeabilization—YO-Pro-1 uptake

The efficiency of electroporation protocols was assessed by the membrane permeabilization assay using Yo-ProTM-1 dye ($\lambda_{\text{exc}} 491/\lambda_{\text{em}} 509$, Thermo Fisher Scientific Inc., Warsaw, Poland). H9c2 cells were trypsinized and suspended in SHM-HEPES-based buffer (2×10^6 cells/mL), and Yo-Pro-1 was added for a final concentration of 1 μM . After pulse delivery, cells were incubated for 3 min at room temperature (RT), followed by the addition of 400 μL of 0.9% NaCl saline solution for measurements in polystyrene fluorescence-activated cell sorting tubes. The samples were excited using the 488 nm line of the blue laser, and the fluorescence of the dye was measured with an FL-1 detector using CyFlow

CUBE-6 flow cytometer (Sysmex, Poland). The control samples without treatment were used as a negative control for gate definition. After permeabilization, depending on the applied protocol, a fluorescent spectrum shift due to dye uptake was observed. Flow cytometric analysis was performed using CyFlow software (Sysmex). The experiments were performed in three technical repetitions.

Cell viability

The viability assay is described in detail in our previous article.¹⁶ After the exposure to the electroporation protocols, cells were seeded in a 96-well plate at a density of 10^4 cells per well. After 24 or 48 h, the absorbance was measured at a wavelength of 570 nm. For measurements, a multiplate reader (GloMax, Promega, Walldorf, Germany) was used. The experiments were performed in triplicate.

Electrotransfer analysis

DNA plasmid vector pEGFP-C1 (Clontech Laboratories, USA) 4731 bp long with DNA coding enhanced green fluorescent protein was propagated in *Escherichia coli* DH5 α strain. Plasmid DNA was extracted from bacteria cells using the alkaline lysis method.¹⁷ The bacteria cells were thoroughly resuspended in 100 μL buffer (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, 100 $\mu\text{g/mL}$ RNase A, pH 8.0). Then, 200 μL lysis buffer (1% SDS, 200 mM NaOH) was added and incubated for 5 min on ice. Proteins were precipitated by adding 150 μL of neutralizing buffer (3 M CH₃COOK, 12 M CH₃COOH) and then separated using centrifugation at 12,000g, 5 min. The clear supernatant was transferred into a fresh tube, and plasmid DNA was precipitated by adding 0.7 volume of isopropanol. After centrifugation at 12 000g per 5 min, the DNA pellet was washed with 500 μL of ethanol, centrifuged, and dried. Plasmid DNA was resuspended in 20 μL nuclease-free water (Merck). The concentration and purity of the plasmid DNA solution were determined using spectrophotometric and electrophoretic analysis. Samples (27 μL) of ice-cold cell suspension and 3 μL of plasmid DNA (2 mg/mL in H₂O) were mixed in a 1.5 mL tube (Eppendorf, Hamburg, Germany) and transferred into an electroporation cuvette with 1 mm gap aluminum electrodes (Biorad, Hercules, CA, USA). The experiments were

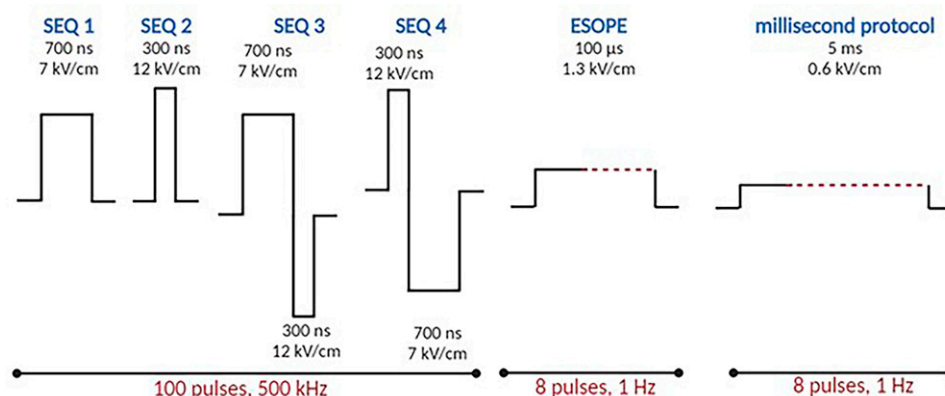


FIG. 1. The patterns of applied sequences of unipolar and bipolar pulses.

performed in triplicate. After pulsed electric field (PEF) protocols, following the 10 min incubation at RT, the cells were suspended in cell medium, transferred into 6-well plates and 35 mm Petri-plates with microscopic glass, and left for 24 h at 37 °C with 5% CO₂. The next day, the cells were analyzed by fluorescent and holotomographic microscopy (3D Cell Explorer microscope, Nanolive SA, Sygnis, Poland).

Statistical analysis

For statistical significance, one-way ANOVA with Dunnett's multiple comparisons test was performed with the GraphPad Prism software, version 7.0. (GraphPad Software, San Diego, CA). The analysis was used for viability results and for the evaluation of fluorescent signals from GET and MAFbx analysis, where differences between treated samples and control cells were taken into consideration. Differences with $p \leq 0.05$ were taken to be statistically significant.

Results and Discussion

To determine the optimal protocols for electrotransfection, first the optimization of the permeabilization protocols was performed (i.e., ensuring a high permeabilization rate and low cytotoxicity). The effects of various nanosecond pulse sequences and the dependence on the electric

field parameters are shown in Figure 2. It can be seen that shorter-duration (300 ns) pulses require higher PEF amplitude to trigger the same permeabilization efficacy when compared with 700 ns bursts; however, equivalent efficiency protocols can be derived. In the case of bipolar pulses (Fig. 2B), a cancellation phenomenon is definitive, especially for bursts starting with a 300 ns positive-polarity pulse. Based on the results, high permeabilization protocols (SEQ 1–4) were selected for each pulse. The permeabilization rate for the ESOPE protocol was $92 \pm 1\%$ and for the millisecond protocol was $85 \pm 2\%$ (data not shown in the graphs). A total of six protocols were defined (Fig. 1). The cytotoxicity of the selected PEF protocols was compared, and the results after 24 and 72 h posttreatment are shown in Figure 2C.

In the next stage, the transfection efficiency of the selected protocols was characterized. As shown in Figure 3A, the UP nsPEF protocol (700 ns) resulted in the highest transfection efficiency. Shorter UP pulses (300 ns) and asymmetric parameters enhanced electrotransfection levels such as ESOPE or millisecond GET protocol. The results agree with our previous studies, where we demonstrated that nanosecond pulses with high repetition frequency can be used for electrotransfection of CHO cells.^{18,19} This study is the first to provide experimental proof of the feasibility

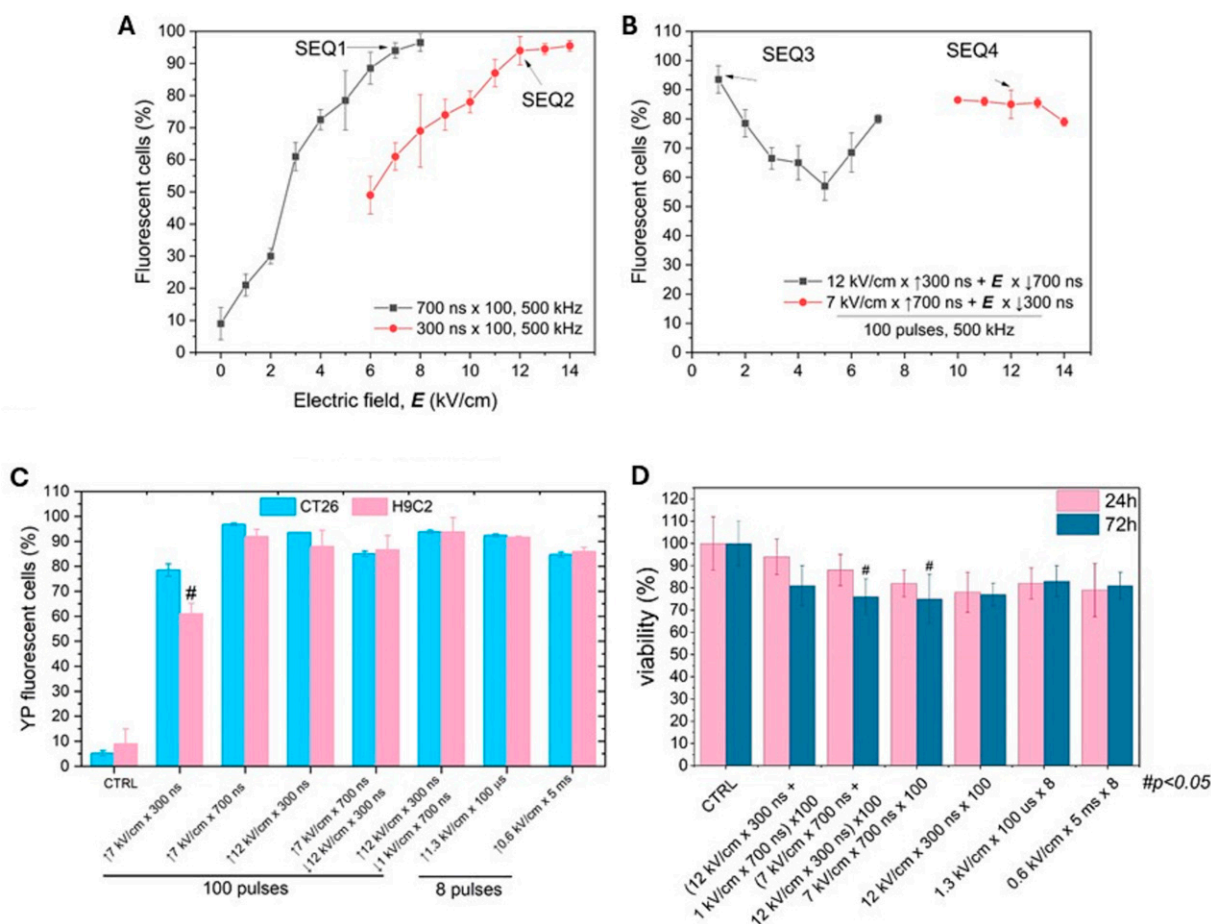


FIG. 2. The efficacy of electroporation determined by Yo-Pro-1TM dye uptake following exposure to different (A) unipolar and (B) bipolar protocols, (C) in comparison to ESOPE and millisecond protocols and CT26 murine cells, and (D) cell viability for the selected high permeabilization protocols determined by MTT assay after 24 and 72 h.

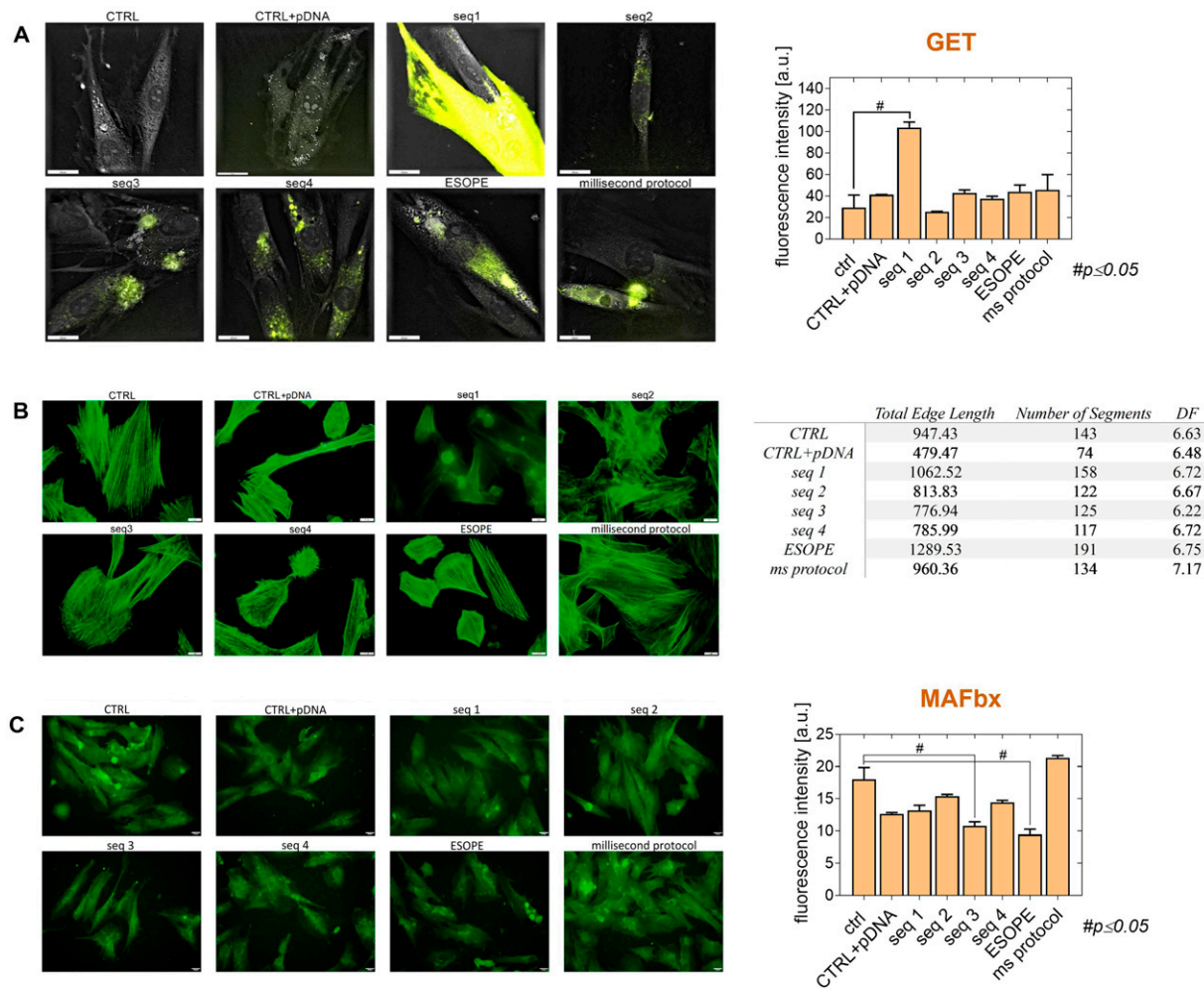


FIG. 3. The analysis of cellular morphology and gene transfer by (A) holotomographic microscopy, (B) the visualization of F-actin organization, and damage factor (table), and (C) the immunofluorescent staining with anti-MAFbx-an atrophy marker and the dependence on PEF parameters. All observations were performed 24 h after exposure to electric pulses.

of nanosecond unipolar and bipolar pulses in the context of electrotransfection of cardiomyocytes.

The available studies provided that the actin cytoskeleton plays a crucial role in the process of DNA electrotransfer, influencing both the accumulation of DNA at the plasma membrane and subsequent gene expression.^{20–22}

In our study, we have performed immunofluorescent staining of actin filaments (see table in Fig. 3B), which shows that unipolar ns protocols induced the most notable and significant cytoskeleton reorganization. Control cells and control with pDNA show a typical cytoskeletal architecture in healthy, non-treated cells. Seq1–4 show varying effects on the F-actin structure, i.e., seq1 (asymmetric pulses) revealed a more disrupted cytoskeleton with diminished stress fibers and more globular actin accumulations, indicating a possible effect on the cell structure or stress response. In the case of seq2, actin fibers appear slightly less organized than in control, with some areas of dense actin accumulation and others where the fibers are less noticeable. The exposure of H9c2 cells to seq3 and ESOPE, induced signs of stress with more dense actin bundles and potential cell shrinkage or contraction. Seq4 induced a notable disruption in the actin

cytoskeleton with areas of intense fluorescence suggesting actin aggregation, and some cells appear to have a rounded morphology, which might indicate a stress reaction. Additionally, we have performed image analysis, using an edge detection algorithm for the F-actin fibers. This enabled us to calculate a damage factor (DF) according to the following equation:

$$DF = \frac{TEL}{No.S}$$

TEL (total edge length) is the sum of the lengths of all detected edges within a panel, and No.S is the number of all connected edge segments detected within a panel. A higher DF would indicate fewer, longer continuous edges, suggesting less damage, while a lower DF would suggest more damage due to more numerous, shorter segments. Our results demonstrated the highest DF, but not significant, in the case of the millisecond protocol, suggesting that the rest of the pulse sequences are safe for cardiac cells. It was also confirmed in viability assay, which may indicate that cytoskeleton changes might be a temporary stress response. Similarly,

the other authors observed that these microsecond pulse effects were voltage-dependent and reversible because cytoskeletal structures recovered within 60 min of electroporation with up to 40 V without any significant loss of cell viability in HUVECs.²³ The other study showed that both the F-actin and β -tubulin of HMEC-1 cells were affected. After EP and ECT occurred, additional granules and spots appeared toward the outer membrane.²⁴ ECT also provoked actin fibers to disappear, and cells shrank or collapsed. In the other research, plasmid DNA delivery by electrotransfection induced cytoskeleton remodeling.²⁵ In the case of nanosecond exposure, induced depolymerization of actin filaments, damage to the nuclear membrane, and telomere damage adversely impact cell survival.²⁶

Finally, we checked the MAFbx gene, a marker for muscular atrophy expressed only in the heart and skeletal muscle. It is believed that loss of muscle expression recognized as atrophy symptoms is also related to the increased expression of MAFbx.²⁷ MAFbx, also known as atrogen-1, is a muscle-specific ubiquitin ligase that is upregulated during muscle atrophy and is a key component of the ubiquitin-proteasome pathway responsible for protein degradation.²⁸ The results are summarized in Figure 3C. Regarding the impact of PEFs on cardiac cells, the decreasing MAFbx signal is more “wanted” as not atrophic. The exposure to seq1 (asymmetric) and seq3 caused a more dispersed distribution of MAFbx, with the fluorescent signal appearing less concentrated than in the CTRL samples. Seq2 also reduced fluorescence intensity, which could indicate a decrease in MAFbx levels. After the exposure to seq4, the signal for MAFbx occurred more unevenly in areas of high intensity. This might suggest localized accumulations or increased expression of MAFbx in certain areas. Our observations indicated that the most common ESOP protocol used in cardiomyocytes was also quite similar to the control, suggesting that this protocol may have less impact on MAFbx distribution than other sequences. The millisecond protocol also appeared to maintain a distribution of MAFbx similar to the control, with a slightly more intense and possibly more even distribution of the fluorescent signal. There, we have also noted that the size of cultured muscle cell lines decreases when MAFbx is overexpressed, suggesting faster protein catabolism, whereas MAFbx gene disruption decreases the loss of muscle caused by nerve transection. Semiquantitative analysis indicates that nsPEF protocols and ESOP diminish the expression of atrophy markers, unlike classical GET protocol using millisecond pulses. Conniff et al. demonstrated that muscle cell manipulation by EP or EP with pDNA can downregulate specific genes, i.e., GPx, which protects cells from oxidative stress, clathrin adaptors that facilitate endocytosis; PDGF, which is involved in cell growth and tissue repair; and also MRFs that regulate muscle development and differentiation.²⁹ These findings might be useful in terms of the PEF application in atrial fibrillation³⁰ or cardiac defibrillation,³¹ where the most important application is minimizing the harmful effects on cardiac contractile properties and improving the efficacy in the arrhythmogenic regions.³²

Our results reveal the ability to utilize UP and asymmetric nsPEF protocols for plasmid delivery to cardiomyocytes. Interestingly, asymmetric pulses in the nanosecond range occurred safe and similarly effective to UP microsecond or

millisecond pulses. This offers new perspectives on the temporal cell membrane permeability conditions for biological vector delivery without cell destruction and protecting against atrophy induction.

Authors' Contributions

J.K. and V.N.: Conceptualization, Methodology, Validation, Writing—Review and Editing Supervision, and Funding acquisition; N.R.: Validation, Investigation, Visualisation, Review and Editing. A.Sz.: Validation, Investigation, Visualisation, Review and Editing. E.R.-V.: Validation, Investigation, Review and Editing.

Author Disclosure Statement

No competing financial interests exist

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