Nanoscale Optoregulation of Neural Stem Cell Differentiation by Intracellular Alteration of Redox Balance


Regulation of stem cell (SC) fate, a decision between self-renewal and differentiation, is of immense importance in regenerative medicine and has been proven to be a powerful stimulus regulating many cell functions influencing the SC fate. This study uses triphenylphosphonium-functionalized gold nanoparticles (TPP-AuNPs) to explore the interplay of intracellular electromagnetic (EM) exposure and the SC fate. Localized EM waves are generated inside neural stem cells (NSCs) to stimulate TPP-AuNPs (AuNPs), targeting the mitochondria through inducing reactive oxygen species and differentiating these cells into neurons. Following laser irradiation of TPP-AuNPs-transfected NSCs, their differentiation to neurons is monitored by tracing the relevant markers both at the genetic and protein levels. The electrophysiology technique is further used to examine the functionality of neurons. The results confirm that TPP-AuNPs subjected to electromotive forces have the potential to regulate cellular fate, although further investigations are still required to shed light on the mechanisms underlying the interaction of EM-stimulated TPP-AuNPs on cellular fate to design highly adjustable cell differentiation and reprogramming methods.

1. Introduction

Stem cells (SCs) are unspecialized cells known for their self-renewal capacity and pluripotency. These cells can proliferate without being differentiated into other cellular types, and are capable of differentiating into a variety of cell lineages.[1] Therefore, SCs are key players in numerous vital processes such as morphogenesis and organ repair.[2] Neural stem cells (NSCs), from three specific niches (the subventricular zone, the subgranular zone of the dentate gyrus, and the external germinal layer of the cerebellum),[3] are multipotent adult SCs with both self-renewing and differentiating properties in development of fetal and adult central nervous system.[4] NSCs can keep their stem cell identity and renew themselves, or can differentiate into other types of cells such as neurons, astrocytes, and oligodendrocytes.[5] Therefore, they have tremendous promise for a variety of cell-based treatments for neural diseases and injuries.[6] However, therapeutic applications of NSCs have been met with only modest success due to the challenge of defining a transplantable NSC population and directing NSC differentiation. Appropriate numbers of NSCs in the nervous system depend on a fine balance between self-renewal and differentiation, something that highlights the importance of controlling the NSC fate as a choice between self-renewal and differentiation.
The balance between self-renewal and differentiation is an important factor for determining NSC fate. Cells use different ways of communication to harmonize their activities. In fact, many cellular functions in SCs rely on both intrinsic and extrinsic signals. Therefore, SC fate can be controlled by interactions between extrinsic signals, derived from the microenvironment in which cells are located (their niche), and intrinsic signals (like transcriptional factors and cell cycle regulators).

Electromagnetic fields (EMFs), in particular, influence intracellular communications via the transport of energy across cells and their microenvironment. EMFs, characterized based on their wavelength and energy, are known as low-level radiations (nonionizing radiations) among the electromagnetic (EM) waves, and do not break atomic bonds. EM stimulations as extrinsic signals have a potential to direct the fate of SCs. According to several reports, the EM nature of biological systems makes the transmission of EM signals possible for the control and regulation of cellular processes. In fact, living cells consisting of biological polar units such as ions are capable of producing EMFs in the form of ultraweak photons. Previous studies have demonstrated the influence of EMFs on some intracellular mechanisms and gene expressions.

Moreover, many studies have reported the influence of externally applied EMFs on cells. The observed effects (like differentiation) are strongly correlated to the cellular or intracellular physiological states and parameters of applied fields such as frequency, intensity, and exposure time. Based on several studies on NSCs, EMFs can promote NSC differentiation into functional cells, for example the differentiating effect of extremely low-frequency electromagnetic fields (ELF-EMFs) on NSCs in rats and embryonic NSCs in mice. Although these studies reported that EMFs can induce differentiation in NSCs, the mechanisms underlying these effects are not well understood. One possible mechanism for the activation of cell differentiation pathways through EM stimulations can be linked to the generation of free radical reactive oxygen species (ROS) and activation of pathways related to ROS. ROS are highly reactive molecules and one site for their generation is mitochondria where they are a byproduct of oxidative phosphorylation. Mitochondrial activities, as a cellular power source, play a regulatory role in cell functions. Therefore, it is reasonable to explore the effects of mitochondria on differentiation of SCs. The aim of this study is to investigate how localized generation of EMFs around mitochondria can regulate the fate of NSC, and explore the feasibility of NSC differentiation through localized EMF.

2. Results and Discussion

A localized EMF is generated around the gold nanoparticles (AuNPs) in the target mitochondria site to examine how EMFs influence the differentiation behavior of NSCs (Figure 1a). Biocompatibility, chemical stability, and adjustable optical properties of AuNPs make them versatile for many biomedical applications. Besides, the possibility of surface modification on AuNPs allow to target specific regions within the cells, which makes the use of these particles a promising candidate for cellular investigations. AuNPs have also been successfully delivered into cells without affecting cell viability and internalized in mitochondria with no reported apoptosis or necrosis.

The interaction of nanoparticles (NPs) with cells starts with the contact with plasma membrane followed by subsequent entry into cells via endocytosis or other means such as pores and channels on the plasma membrane. This complex interaction is highly dependent on the size, shape, and surface property of NPs. After internalization, it is necessary to direct NPs to their specific target within cells. Conjugation of cationic groups to the surface of AuNPs results in water-dispersed NPs. Triphenylphosphonium (TPP) is a lipophilic phosphonium cation with a high affinity to mitochondria. TPP cations have been used for studying mitochondrial bioenergetics and free radicals. In this study, the AuNPs were functionalized with the phosphonium groups (TPP cations) for mitochondrial localization. The attachment of AuNPs to the TPP cations produced a mechanism targeting the mitochondria followed by their accumulation within the cells. This system enables studying the intracellular effects of localized EMFs by laser irradiation.

AuNPs were synthesized as detailed in the Supporting Information, functionalized with TPP, and analyzed with UV-vis spectrophotometry, high resolution transmission-electron microscopy (HR-TEM), and dynamic light scattering (DLS). The HR-TEM analysis of the AuNP-TPP (Figure 1b) shows spherical and monodispersed functionalized NPs, corroborated with the size distribution analysis of particles using DLS, presenting an average particle size of ≈10 nm (Figure 1c). Particles with the size of 10 nm are selected in this study given their acceptable level of cellular uptake and the ease of tracing them inside the cells. The UV–vis absorption measurement of AuNPs (Figure 1d) illustrates the maximum absorption wavelength at 529 nm corresponding to the localized surface plasmon resonance of AuNPs, shifted to 520 nm after TPP conjugation. The refractive index around the particles changes after TPP binding, which consequently causes the shift in the wavelength of absorption peak, confirming the strong binding of TPP to the surface of AuNPs (Figure 1d). The successful conjugation of TPP on the surface of AuNP-Amine is also confirmed using 1H NMR (Figure S1, Supporting Information). It was demonstrated that the surface density of TPP on the surface of AuNPs can be controlled by changing the concentration of TPP in the reactant mixture.

The absorption cross-section, $\sigma_{abs}$, of the light-stimulated AuNPs is the equivalent surface area required to absorb the same amount of power in the absence of NPs. NPs typically have absorption cross-sections larger than their dimensions because of the localized surface plasmon polariton resonances. Increasing the size of nanospheres from 10 to 14 nm does not change the resonance wavelength considerably ($\lambda_{res}$ ≈ 527 nm) but increases $\sigma_{abs}$ significantly (Figure 1e). As an example, a NP with a radius of 5 nm has a $\sigma_{abs}$ of ≈ 71.84 nm$^2$ at 540 nm. Assuming the exposure of cells to 1 mW of laser power over a spot size diameter of 2 µm, the power of ≈ 22.87 pW is absorbed per NP (Figure 1f–h).

The intracellular localization and distribution of NPs were investigated by confocal laser scanning microscopy (CLSM). Figure 2a–d illustrates the fluorescent images of incubated...
NSCs with nonfunctionalized AuNPs (Figure 2a,c) and functionalized AuNPs (Figure 2b,d) for 4 h at 37 °C. The green fluorescence image is related to the fluorescein isothiocyanate isomer labeled NPs (panel (i)), while the red fluorescence image indicates MitoTracker (panel (ii)). Panel (iii) with yellow spots shows the merged images of green and red, demonstrating the mitochondrial localization of NPs. The degree of colocalization is also demonstrated in Figure 2b,d for cells incubated with AuNPs and TPP-AuNPs, respectively. The confocal images reveals the high level of NPs internalization in NSCs and the successful colocalization of AuNP-TPP in mitochondria, concluded from yellow fluorescence signals in panel (iii). The higher association between the red and green fluorescence channels, represented in intensity-based 3D reconstruction of fluorescent images (Figure 2d), also confirms a successful colocalization with TPP-AuNPs in comparison with unmodified AuNPs.

Additional spectrometric assays were performed to confirm and quantify the presence of TPP-AuNPs inside the cells. Cellular internalization of AuNPs and TPP-AuNPs were measured by the fluorescence activated cell sorting method (Figure 2e(i)) and inductively coupled plasma mass spectrometry (Figure 2e(ii)). Although surface functionalization with TPP results in a slight decrease in cellular uptake compared to nonfunctionalized NPs, a high number of internalized NPs are observed in both functionalized and nonfunctionalized NPs. However, mitochondrial uptake, as quantified by mitochondria isolation, confirms a higher targeting efficiency of 75% for AuNP-TPP compared to 30% for AuNPs after 4 h coculturing.

The examination of TPP-AuNPs cytotoxicity as one critical step after internalization was performed by the MTT (3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide) assay. The viability of cells in the presence of AuNPs and TPP-AuNPs with and without irradiation (see the Supporting...
Figure 2. Mitochondrial targeting efficiencies. Confocal laser scanning microscopy images on mitochondrial colocalization of a,c) AuNP and b,d) AuNP-TPP. Panels (i)–(iii) indicate the mitochondria stained with MitoTracker (red), AuNPs labeled by fluorescein isothiocyanate (FITC) (green), and the overlay image of them (yellow) (scale bar is 20 µm). e) Cellular uptake of fluorescently labeled AuNP and AuNP-TPP using fluorescence-activated cell sorting (FACS) (left panel). Cellular uptake evaluated using inductively coupled plasma-mass spectrometry (ICP-MS) spectroscopy. Mitochondrial uptake portions are indicated within each bar. *p < 0.05 and **p < 0.01 between cellular/mitochondrial uptake after 4 and 1 h for AuNP and AuNP-TPP. ##p < 0.01 between mitochondrial uptake for AuNP-TPP and AuNP. f) Cellular viability of NSC after 72 h incubation with AuNP and AuNP-TPP w/o irradiation. *p < 0.05 between each treatment group and cells treated with AuNP without irradiation (control (−)). g) In vial generation of reactive oxygen species (ROS) after laser irradiation. h) Monitoring of intracellular ROS generation during 120 min of treatment with laser in the presence and absence of AuNPs. The empty triangles indicate the irradiation points. *p < 0.05 and **p < 0.01 between the sample with and without irradiation (negative controls).
Information for details) was measured, and statistically compared among various concentrations of NPs from 0.25 to 40 µg mL\(^{-1}\) (Figure 2f). It seems that the viability of all treated cells (AuNPs-TPP and AuNPs-Amine with/out irradiation) are quite high (>95%) after the incubation with low concentrations of NPs (up to 1 µg mL\(^{-1}\)). The results also indicate that the surface functionalization of NPs has a trivial impact on the toxicity of NPs. The increase in the concentration of AuNPs from 0.25 to 40 µg mL\(^{-1}\) causes a significant cellular toxicity, especially in the presence of irradiation. The irradiated cells in both groups, incubated with AuNPs and TPP-AuNPs, show a lower cell viability compared to nonirradiated cells. A plausible explanation for the reduction in the cell viability is the involvement of a higher production rate of ROS. The excessive concentration of ROS can lead to oxidative stress and cell death.[39,40] The results indicate that the fraction of dead cells increases for cells loaded with high concentrations of NPs (above 5 µg mL\(^{-1}\)) while the cell viability is above 90% at low concentrations of NPs (up to 5 µg mL\(^{-1}\)). The dose-dependent results confirm that the cellular toxicity of NPs is trivial even after 1-hour irradiations at concentrations below 2 µg mL\(^{-1}\). It should be noted that the optimal cell viability (>95%) was achieved by setting the concentration of NPs to 1 µg mL\(^{-1}\), even in the presence of irradiation selected for further experiments.

Despite the destructive effects of ROS, these active species can act as the second messenger in a wide variety of biological processes.[38,39] A growing body of evidence indicates that ROS have mediatory roles in cell growth, proliferation, and differentiation.[39,40] The implication of ROS as the second-messengers in maintenance and growth of cells has been demonstrated for NSCs.[41] Interestingly, it has been reported that ROS can influence NSC differentiation and regulate neurogenesis,[41,42] EMFs affect ROS generation as a factor influencing the SC fate. The stimulation of NADH (nicotinamide adenine dinucleotide—hydrogen (reduced))-oxidase pathway and ROS production in mouse bone marrow-derived cells after applying extremely low frequency magnetic field (ELF-MF) (50 Hz, 1 mT); enhanced production of ROS in murine L929 fibrosarcoma cells exposed to the radiofrequency EMFs (at 900 MHz); increased ROS level in the rat lymphocytes under treatment with a combination of radiofrequency radiation and iron ions; and elevated ROS level and enhanced neural differentiation of human MSCs exposed to ELF-EMF (50 Hz, 1 mT), are some evidence of the EMF impact on ROS generation. It should be noted that EMFs can also influence the activation of antioxidant enzymes and prevent ROS generation. In rat’s liver mitochondria, for example, the generation of free radicals was prevented upon the activation of antioxidant enzymes through EM pulse exposure (60 kV m\(^{-1}\) strength). An increase in the activity of SOD (superoxide dismutase) enzymes after the exposure of mice and rat brains, respectively, to the ELF-magnetic field of 60 and 50 Hz are other indicators of the effect of EMF on antioxidant activities.[42] In a study on embryonic NSC, the messenger ribonuclease acid (mRNA) level of genes related to neuronal differentiation was affected after the treatment of cells with 50 Hz ELF-EMF (2 mT for 3 d).[43]

On the other hand, mitochondrial activity and the amount of ROS generation are closely related.[10] The rate of mitochondrial activity is higher in undifferentiated NSCs in contrast to differentiated neurons and glial cells while the rate of ROS generation is less.[46] It is, therefore, suggested that the intracellular redox balance may be influenced after laser irradiation, and ROS may serve as a regulating factor in differentiation of NSCs.

To examine the effect of laser irradiation on intracellular redox state, the cellular ROS value was measured after laser irradiation. To detect ROS formation, the fluorescence intensity of dihydrorhodamine 123 (DHR123), as a ROS tracking agent, was measured using fluorescence spectrophotometry. Figure 2g shows the increase in the fluorescence intensity with irradiation time, indicating that the ROS concentration can be adjusted by tuning the irradiation time. Also, Figure 2h shows the increase in the intracellular ROS concentrations within the cells after laser irradiation. The detected decline in the plateau level of the ROS intensity occurring after each peak in the graph can be attributed to the short lifetime of ROS and the activity of various redox systems inside the cells (like antioxidants[47] having a suppressive effect on the ROS), which balances the cellular oxidative stress.[26]

The reverse transcription polymerase chain reaction (RT-PCR) technique was further employed to investigate neural differentiation of NSCs after treatment with NPs followed by laser irradiation. The RT-PCR data shows that NPs and laser irradiation induced differentiation of NSCs via altering the expression levels of Nestin, β-tubulin III, myelin basic protein (MBP), Olig2, and Glial fibrillary acidic protein (GFAP), as representative marker genes for neural stem/precursor cells, neuron, oligodendrocyte, and astrocytes, respectively. It is well-known that upon differentiation of NSCs, the expression of Nestin, a marker for undifferentiated NSCs, decreases progressively and the level of neuronal differentiation markers increases,[48] implying the production of more mature cells, in agreement with RT-PCR data (Figure 3).

At three different stages (day 1, 7, and 14), cells from different groups (treated with or without laser irradiation and TPP-AuNPs/AuNPs) were processed for gene expression analyses. The expression of each gene was plotted in Figure 3a–d and Figure S4 (Supporting Information) after normalizing to the expression of glyceraldehyde-3-phosphate dehydrogenase mRNA. For negative control group, the cells were cultured in normal culture medium without any treatment with NPs and irradiation. For positive control group, the cells were cultured in differentiation medium containing neurobasal medium with B-27 serum-Free supplement and Glutamax-I to induce neural differentiation. Under the negative control condition, NSCs express a high level of Nestin while neuronal differentiation markers (β-tubulin III, MBP, Olig2, and GFAP) stay at their lowest levels. As determined by RT-PCR, the expression of β-tubulin III, MBP, Olig2, and GFAP is up-regulated within two weeks of differentiation, and the expression of Nestin decreases in the differentiation groups compared to negative control.

The effect of laser irradiation on up-regulation of the differentiation markers like β-tubulin III, MBP, and GFAP and down-regulation of the Nestin level are shown in Figure 3a–d. More importantly, the results indicate that changes in the expression of markers are most conspicuous when NSCs are treated with both NPs and laser irradiation. The exposure to laser irradiation increases the gene expression of β-tubulin III, MBP, Olig2,
and GFAP and significantly decreases the Nestin expression, hinting at the potential of irradiation for differentiating NSCs into neurons, astrocytes, and oligodendrocytes. After a two week treatment with NPs and irradiation, a clear difference are observed in the expression of neuronal markers between the irradiated and nonirradiated cells containing NPs, which indicates that the low power laser irradiation (here 1 mW) has a strong impact on NSC differentiation. Note that the effect of irradiation is more prominent on the marker expression in the groups of cells loaded with TPP-AuNPs compared to the cells containing nonfunctionalized AuNPs. The expression of Nestin in irradiated NSCs containing TPP-AuNPs shows no significant difference respect to those of the positive control group with five times less expression of Nestin than the negative control group (Figure 3c). By contrast, the neuronal differentiation markers in the cells irradiated for two weeks show a significant up-regulation compared to the nontreated group, especially β-tubulin III and Olig2 expressed one-fold more than the negative control. The N-Acetyl-L-cysteine as a ROS scavenger was also used to confirm whether ROS generation using the laser
irradiation is the cause of the proposed biological effects. As shown in Figure S2 in the Supporting Information, the ROS scavenger can eliminate ROS signals in vial. Evaluating the RT-PCR results of β-tubulin III and Nestin expression compared to housekeeping gene (GFAP), it is found that the inhibition of ROS generation can prevent neural differentiation as expected (Figure S3, Supporting Information).

Western blotting assay and CLSM were used to verify the laser irradiation effect on NSC differentiation in the presence of TPP-AuNPs. In agreement with the RT-PCR results, the protein expression assay demonstrates the neural differentiation potential of NSCs containing NPs after the irradiation in which the cells are labeled for β-tubulin III and GFAP. The protein expression plot for β-tubulin III shows the increasing number of β-tubulin III positive cells within 14 d after treatment with TPP-AuNPs in both groups of irradiated and nonirradiated, like what detected for the control group with normal neurodifferntiation treatment. As depicted in Figure 3f, the number of β-tubulin III positive cells from the irradiated group is approximately twice the number of positive cells in the nonirradiated group. After 14 d, the number of β-tubulin III positive cells reaches about 20% of the total cells in the irradiated group while that is close to the control group with normal neural differentiation treatment (See Supporting Information for more details). A similar trend is observed within 14 d for the GFAP expression. However, there is no significant difference between the irradiated, nonirradiated, and control groups after 14 d. Overall, the protein expression plots show increased percentages of GFAP positive and β-tubulin III positive cells after irradiation of NSCs containing NPs, confirming the differentiation of NSCs into neurons and astrocytes. Notably, NSCs containing NPs show more tendencies toward glial differentiation with or without irradiation. Also the incidence of neuronal differentiation is also supported by the CLSM image of NSCs containing TPP-AuNPs after laser irradiation at 1 mW (Figure 3g). The cells are stained with antibodies against GFAP (green) and β-tubulin III (red) while the nuclei are counterstained with 4’,6-diamidine-2’-phenylindole dihydrochloride (blue). Both GFAP and β-tubulin III are detected in CLSM images, confirming the presence of GFAP+ and β-tubulin III+ cells, and the differentiation of cells at day 14.

To assess the functionality of differentiated cells, their neural activity was examined by measuring their electrophysiological properties, including the membrane currents and Ca2+ concentration. First, the presence of the voltage-gated channels was confirmed by using patch clamp whole-cell recording as described before.[49] In voltage-dependent currents, depolarizing current injection using patch clamp whole-cell studies after stimulation of the differentiated cells with the voltage pulse stimuli open the Ca2+ channels and increase extracellular Ca2+ concentration, the cells were electrically stimulated as described before.[50] The current–voltage relationship of sodium (Na+) and potassium (K+) currents in cells undergoing 7 and 21 d terminal differentiation show remarkable inward Na+ current with a mean peak of approximately −870 ± 50 pA at −20 mV for the second stage of maturation (day 21) (Figure 4a). The I–V relationship of the potassium (K+) current at two stages of maturation demonstrates an outward current (Figure 4b). These voltage-dependent Na+ and K+ currents in the stimulated cells indicate that voltage-sensitive ion channels exist in the differentiated cells, confirming the presence of physiologically active neuronal cells. However, NSCs treated with AuNP-TPP without irradiation fail to show the same level of voltage-dependent Na+ and K+ currents (Figure S5, Supporting Information).

Next, calcium imaging experiments were performed to monitor the change of Ca2+ level as a physiological marker of differentiation inside the differentiated cells. To verify intracellular Ca2+ concentration, the cells were electrically stimulated as described before.[50] The calcium imaging represents a transient calcium increase in the stimulated cells (Figure 4c), which is quantified in Figure 4d. Based on the plot of the relative change (ΔF/F) of the cell fluorescence intensity versus the stimulation period, over 60% increase in the fluorescence intensity is observed after the stimulation, corroborating an increased intracellular Ca2+ concentration. This result implies the appearance of voltage-sensitive Ca2+ channels in the differentiated cells, which is a basic neuronal membrane characteristic. Thus, the voltage pulse stimuli open the Ca2+ channels and increase the Ca2+ concentration in the differentiated cells.[51] Overall, this study show that laser irradiation significantly increases the expression of neuronal differentiation genes in the presence of NPs, emphasizing the potential of EM exposure to enhance neuronal differentiation of NSCs.

Although researchers are struggling to bring up the mechanisms underlying EM exposure for SC differentiation, some reports have suggested the probable relationship between ROS production and underlying mechanisms of EM exposure for differentiation. Additionally, the relationship between ROS generation and mitochondrial activity of differentiated and undifferentiated NSCs suggests the control of SC fate through controlling ROS generation. Our results also show that ROS production increases in NSCs after the exposure to laser irradiation. It seems reasonable to suggest that ROS production is involved in the mechanisms underlying the interaction of EMF and NSCs.

It should be highlighted that EMF may cause differentiation in NSCs through the activation of ionic channels and alteration in ion concentration,[32] changes in gene expression (transcriptional factors and pluripotency markers),[33] and influencing intracellular molecular mechanisms such as MAPK (mitogen-activated protein kinase) pathway.[14] However, the underlying mechanisms of EM exposure responsible for neural differentiation of NSCs is still unclear and need to be further clarified. Undoubtedly, further experiments are needed to better understand the redox-dependent mechanisms for NSC differentiation in response to laser irradiation. In our experiments, although EM exposure induced differentiation in NSCs in the presence of NPs, the differentiation was non-selective. One major challenge would be the differentiation of NSCs into specific neuronal and glial cell lines. There is a need to investigate whether the combination of NPs and laser irradiation treatments could guide the NSCs differentiation toward specific cell types, and whether ROS-related mechanisms work differently in astrocyte, neuron and oligodendrocyte differentiation to present a selective protocol for the differentiation of NSCs. However, our approach has been utilized as a technique (nanoparticles and stimulatory EM generation) to promote the differentiation of NSCs in the absence of soluble cues.
This methodology can also potentially be helpful to investigate how localized generation of EMFs in a targeted site (mitochondria or cell membrane) can influence cell responses to explore the feasibility of NSC differentiation through localized EMF. Furthermore, our proposed system can be used as a research tool to study the intracellular effects of localized EMFs within the cells and identify genes and signaling pathways effective in regulation of NSC fate. This system can also be translated into better understanding how NSCs balance self-renewal versus differentiation to address an optimal treatment for neurodegenerative diseases like Alzheimer’s and Parkinson’s. Although we focused on NSCs differentiation, this technique could be extended to study and control the fate of other stem cells, from a variety of types, stages of differentiation and sources.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

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