Ginkgo Biloba Extract Attenuates Oxidative Stress and Apoptosis in Mouse Cochlear Neural Stem Cells

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INTRODUCTION

Hearing loss has become a worldwide health problem, especially in aged population (Seidman et al., 2002), and the molecular basis of age-related hearing loss was largely considered to be due to oxidative stress (Yamasoba et al., 2013). Findings from several reports collectively suggested that in aged population, hearing loss can be triggered by the excess production of reactive oxygen species (ROS) in the blood flow of the cochlea (Bielefeld et al., 2010; Fetoni et al., 2011; Fujimoto and Yamasoba, 2014), where the auditory hair cells are located in mammalian inner ear. ROS are mainly produced by the mitochondria in mammalian cells (Turrens, 2003). Although they are often seen as toxic metabolic byproducts, they were recently found to function as signaling molecules that regulate many physiological processes (Sena and Chandel, 2012), including apoptosis (Sinha et al., 2013).

Neural stem cells (NSCs) can self-renew, proliferate, and in turn differentiate into neurons and glial cells (Gage, 2000). Following neuronal damage, NSCs can be activated and migrate to the injured sites to replace the lost neurons; therefore, they hold great therapeutic potential in the treatment of neural damage and neurodegenerative diseases (Nakatomi et al., 2002; Russo et al., 2011). In mammals, hearing loss can be caused by primary degeneration of spiral ganglion neurons or by secondary degeneration of neurons. Therefore, replacement of these neurons is the first step to restore sensory neuronal hearing loss. We have demonstrated earlier that inner ear NSCs are capable of forming neurospheres in vitro and differentiate into functional neurons (Wang and Han, 2015), and successful cochlea transplant derived from inner ear NSCs has also been reported recently (Hu et al., 2005). However, poor survival rate of transplanted stem cells are a major obstacle in NSC-related therapies, because of the hostile environment usually caused by oxidative stress. For example, exposure to hydrogen peroxide, a strong oxidant reagent, caused severe loss of cell viability in culture rat NSCs (Zhang et al., 2015). Oxidative stress also led to a marked reduction in cell viability of adult spinal cord—derived neural stem/progenitor cells (Hachem et al., 2015).

Ginkgo biloba extracts (GBE), retrieved from Chinese herbal medicine, contain 22–24% Ginkgo flavonoids and 5–7% terpen triactones (gingolides, bilobalide). EGb761 contains ginkgolides A, B, C, and bilobalide and is the main ingredients of GBE (EGb 761: ginkgo biloba extract, Ginkor, 2003) (Fig. 1A). GBE was found to exhibit free radical scavenger activity, prevent high glucose-induced diabetic cataract, and has neuro-protective and anti-apoptotic properties (Diamond et al., 2000; Kang et al., 2007; Lu et al., 2014). Particularly relevant to our current study, GBE was reported by several studies to exhibit protective effects on the survival of mammalian stem cells. For instance, GBE promotes survival of rat bone-marrow mesenchymal stem cells upon ischemia both in vitro and in vivo (Li et al., 2011). It also enhances proliferation of NSCs in the subventricular zone and dentate gyrus and significantly improves learning and memory in rats with vascular dementia (Wang et al., 2013). Moreover, GBE also

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exhibited anti-apoptotic effects by inhibiting apoptosis induced by high glucose in human lens epithelial cells (Wu et al., 2008), as well as anti-oxidative effects by protecting against lead-induced oxidative stress in specific regions of rat brain (Yallapragada and Velaga, 2015).

In this study, we aimed to investigate whether the reported anti-oxidant and anti-apoptosis effect of GBE could be applied in the protection of cochlear NSCs against oxidative stress. In the isolated mouse cochlear NSCs culture, we used hydrogen peroxide to establish an in vitro oxidative stress model and assessed the effect of GBE in protecting NSCs upon hydrogen peroxide challenge. We found that GBE promoted cell viability, which was mediated by markedly attenuating H₂O₂-induced oxidative stress in the NSC culture. GBE also prevented mitochondrial depolarization and subsequent apoptosis. We further found that the anti-apoptotic role of GBE was mediated by antagonizing the intrinsic mitochondrial apoptotic pathway. Our study provided the first evidences on the beneficial role of GBE in protecting mammalian cochlear NSCs.

MATERIAL AND METHODS

Ethics statement and mouse cochlea isolation. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by Eye and ENT Hospital of Fudan University. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize animal suffering. Isolation and culture of mouse cochlea were performed using previously described method (Wang and Han, 2015). Briefly, early postnatal Balb/c mice were decapitated, and the temporal bone was dissected out and transferred into ice-cold Hank’s balanced salt solution (Invitrogen). The otic capsule was freed from the otic bulla, opened, and removed to visualize the membranous labyrinth of the cochlea. The organ of Corti was micro-dissected from the modiolus where the spiral ganglion resides.

Isolation and Culture of cochlear neural stem cells. Mouse cochlear NSCs were isolated using previously described method (Diensthuber et al., 2009) Briefly, spiral ganglia were enzymatically digested in 0.125% trypsin/EDTA in phosphate-buffered saline (PBS) for 5 min at 37 °C. Digestion was terminated by a combination of 10 mg/mL soybean trypsin inhibitor and 1 mg/mL DNase I ( Worthington) in Dulbecco’s modified Eagle’s medium and Nutrient Mixture F12 (DMEM/F12; Sigma) followed by trituration with mechanical dissociation to obtain a homogeneous single cell suspension. The cells were then cultured in poly-HEMA-coated suspension culture six-well plates (Sigma) in a volume of 2 mL of DMEM/F12 supplemented with N2 (Invitrogen), B27 (Invitrogen), 20 ng/mL epidermal growth factor, 10 ng/mL basic fibroblast growth factor, 50 ng/mL insulin-like growth factor-1 (IGF-1), and 50 ng/mL heparan sulfate (all growth factors from Sigma-Aldrich), 50 μg/mL ampicillin.

Figure 1. Ginkgo biloba extract (GBE) improved cell viability following H₂O₂ treatment in mouse cochlear neural stem cells (NSCs). (A) Ginkgolid A, B, C and bilobalide are two main ingredients of GBE. (B and C) Relative cell viability of the NSCs to the control following GBE (B) or H₂O₂ treatments, respectively. (D) Relative cell viability of the NSCs to the control, following incubation with medium only (control), 0.25 μM H₂O₂ only, 50 mg/L GBE only or a combination of 0.25 μM H₂O₂ and 50 mg/L GBE at indicated times. Data were presented by the mean ± SEM from three independent experiments. *p < 0.05 compared respective control group; #p < 0.05 compared H₂O₂ only group.
Hydrogen peroxide and ginkgo biloba extracts treatment. Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) was used in the current study to induce oxidative stress, which was supplemented in the media at assay-specific concentrations. GBE (Pizhou City, China) was supplemented in the media at an optimal concentration of 50 mg/L as previously described (Wang and Han, 2015). Media supplemented with both 0.2 mM H\textsubscript{2}O\textsubscript{2} and 50 mg/L GBE was used as treatment group, with the same media without H\textsubscript{2}O\textsubscript{2} or GBE serving as negative control group. All media were exchanged every day to ensure the availability of H\textsubscript{2}O\textsubscript{2} and GBE in the culture.

Cell viability assay. Cells grown in 96-well plates were treated with H\textsubscript{2}O\textsubscript{2} and/or GBE as indicated in respective figures for 24 h. Ten microliter of MTT (5 mg/mL) was added to each well, and the plate was incubated at 37 °C in the dark for 4 h. Then, the media along with MTT was removed, and the formazan crystals were solubilized by adding DMSO (100 μL/well). Finally, the reduction of MTT was quantified by reading the absorbance at 570 nm by microplate reader.

Cellular reactive oxygen species measurement. Cellular ROS level was measured by DCFH-DA (Life Technologies) according to manufacturer’s instructions. Briefly, cells were washed twice with pre-warmed serum-free DMEM and incubated with 5 μM DCFH-DA in serum-free medium for 30 min, followed by washing with PBS. Fluorescent signals were quantified from nine randomly picked regions of interest from three independent experiments.

Oxidative stress measurements. Oxidative stress was determined by measuring the activity of superoxide dismutase (SOD), the level of glutathione (GSH), and malondialdehyde (MDA). SOD activity was measured by Superoxide Dismutase Assay Kit (Cayman Chemical, UK) following the manufacturer’s instructions. Briefly, cell lysis was mixed with the reaction mixture followed by initiation with NADH and termination with glacial acetic acid, and absorbance at 560 nm was measured by a microplate reader. GSH levels were measured using the GSH Assay Kit (Cayman Chemical). Briefly, cell lysis was mixed with 5, 5-dithiobis-(2-ni-trobenzoic acid) (DTNB), and phosphate buffer and absorbance at 412 nm was measured by a microplate reader. MDA levels were measured using previously described method (Buege and Aust, 1978). Briefly, cell lysis was heated with thiobarbituric acid at 95 °C for 40 min, then centrifuged at 3500 rpm for 10 min. The supernatant was collected, and absorbance at 532 nm was measured by a microplate reader.

Mitochondrial transmembrane potential measurement. Mitochondrial transmembrane potential is measured as described previously (Kumar et al., 2008). Briefly, cells were washed twice with pre-warmed serum-free DMEM and incubated with 2.5 μg/mL JC-1 (Life Technologies) for 30 min, followed by washing with PBS. Fluorescent signal was analyzed on a flow cytometer with 530 nm and 590 nm band pass emission filters. Mitochondrial depolarization was indicated by the ratio of the green/red fluorescence (530/590).

Apoptosis measurement. Measurement of apoptosis was performed by Annexin V staining according to the manufacturer’s instructions (Beckman Coulter, France). Cells were stained with Annexin V-FTIC and then were washed with PBS for three times. The stained cells were analyzed on a Beckman Coulter FC500.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from NSCs using the RNasy MiniPrep Kit (Qiagen). One microgram of total RNA was reverse-transcribed with SuperScript II First-Strand Synthesis kit (Life Technologies) as recommended by the manufacturer. Actin mRNA levels were measured for normalization, and all data were presented as relative expression.

Western blot. Cell lysate was added into 20 μL 2× sample loading buffer (0.125 M of 5 M Tris–HCl, Amresco; 20% glycerol, Usb; 4% of 10% sodium dodecyl sulfate, Amresco; 1% β-mercaptoethanol, Amresco; 0.2% of 0.05% (w/v) bromophenol blue, Sigma) and boiled for 5 min before loading. Proteins were separated by SDS-PAGE, transferred to immobilon P membrane (Millipore), and were probed with antibodies against Bax, Bcl-2, Caspase-3 and Actin as indicated. All antibodies were purchased from Cell Signaling. The results were visualized using ECL kit (Abcam).

Statistical analysis. Data were presented by the mean ± SEM from three independent experiments. Statistical analysis between groups was performed by two-way Student’s t test, p values < 0.05 were considered significant. Post hoc test was performed only if F value < 0.05, and there was no significant variance inhomogeneity (Curtis et al., 2016).

RESULTS

Ginkgo biloba extract improved cell viability following H\textsubscript{2}O\textsubscript{2} treatment in mouse cochlear neural stem cells

First of all, mouse cochlear NSCs were isolated as described in Materials and Methods section, and their stemness was confirmed as previously described (Wang and Han, 2015). We have already demonstrated that Egb761, a standardized and well-defined product of Ginkgo biloba extract, increased cell viability of isolated mouse cochlear NSCs in vitro as measured by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Wang and Han, 2015).

In the current study, we first confirmed our earlier findings where mouse cochlear NSCs cultured in media supplemented at with 50 mg/L GBE for 24 h exhibited optimal improvement in cell viability (Fig. 1B). Next, we employed H\textsubscript{2}O\textsubscript{2} as the means to induce oxidative stress in the culture (Fig. 1C), by incubating the NSCs with media containing increasing concentrations of H\textsubscript{2}O\textsubscript{2} (0.25, 0.5, and 1 μM). After 24 h, viability of NSCs reduced to approximately 25% of the control in media containing 0.25 μM H\textsubscript{2}O\textsubscript{2}, whereas it was further reduced to less than half of control in the case of 0.5 and 1 μM H\textsubscript{2}O\textsubscript{2}. In order to assess whether GBE was able to protect the mouse cochlear NSCs from H\textsubscript{2}O\textsubscript{2}-induced loss of viability, we also treated the cells with medium
only as control, 0.25 μM H₂O₂ only, 50 mg/L GBE only, and a combination of 0.25 μM H₂O₂ and 50 mg/L GBE, followed the growth of cells and measured their viability after incubation in respective media for 12, 24, and 36 h (Fig. 1D). Compared with the control at time 0, treatment with H₂O₂ alone exhibited a time-dependent inhibition on cell viability, whereas GBE alone at the same time points consistently promoted cell viability over that of control. More importantly, the combination of H₂O₂ and GBE was able to improve NSC viability significantly over that of H₂O₂ treated culture throughout the experiment. Particularly at 24 h, viability of NSCs in the H₂O₂ + GBE treatment group was even significantly higher than the control group, indicating the optimal incubation time.

Ginkgo biloba extract attenuated H₂O₂-induced oxidative stress in mouse cochlear neural stem cells. As exposure to H₂O₂ was widely known to cause the production of cellular ROS, we next investigated whether the observed attenuation of GBE on H₂O₂-induced loss of viability was mediated through reducing oxidative stress. Similarly mouse cochlear NSCs were treated with medium only as control, 0.25 μM H₂O₂ only, 50 mg/L GBE only, or a combination of 0.25 μM H₂O₂ and 50 mg/L GBE for 24 h, followed by DCFH-DA staining to determine cellular ROS production (Fig. 2A). As expected, H₂O₂ only treatment resulted in a significant increase in ROS production in the culture, compared with the control group. Whereas ROS production in the H₂O₂ + GBE treatment group was significantly reduced than the H₂O₂ only group, indicating that the addition of GBE in the H₂O₂-treated culture attenuated ROS production. GBE treatment alone did not exhibit any significant effect on ROS production.

Besides ROS, the level of GSH, the activity of SOD, and MDA production were also commonly used as indicators of oxidative stress; therefore, we measured these parameters to further validate the anti-oxidative effect of GBE in the mouse cochlear NSC culture. As shown in Figs. 2B and C, GSH level and SOD activity in the H₂O₂-treated cultures were significantly decreased that of those of respective control groups. Moreover, the production of MDA in the H₂O₂-treated cells was also significantly increased compared with control (Fig. 2D). These results indicated that H₂O₂ strongly induced oxidative stress in the in vitro cultured mouse cochlear NSCs. Importantly, simultaneously treatment with H₂O₂ and GBE could fully restore GSH level, SOD activity and MDA production to almost the same levels as their respective controls, suggesting that GBE indeed could significantly attenuate H₂O₂-induced oxidative stress. Taken together, the aforementioned results strongly demonstrated the robust anti-oxidative effect of GBE in mouse cochlear NSCs.

Ginkgo biloba extract attenuated H₂O₂-induced mitochondrial depolarization and apoptosis in mouse cochlear neural stem cells. Oxidative stress often causes the opening of mitochondrial permeability transition pores and in turn mitochondrial depolarization (Chirichigno et al., 2002). Consistent in our current study, we found that in mouse cochlear NSCs, H₂O₂ exposure significantly increased mitochondrial depolarization (Fig. 3A), and H₂O₂ + GBE combined treatment was able to restore the H₂O₂-induced mitochondrial depolarization. GBE treatment alone did not exhibit any significant effect on ROS production.

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**Figure 2.** Ginkgo biloba extract (GBE) attenuated H₂O₂-induced oxidative stress in mouse cochlear neural stem cells. Mouse cochlear neural stem cells were treated with medium only (control), 0.25 μM H₂O₂ only, 50 mg/L GBE only, or a combination of 0.25 μM H₂O₂ and 50 mg/L GBE for 24 h, followed by measurement of cellular ROS levels (A), glutathione (GSH) levels (B), superoxide dismutase (SOD) activity (C) and malondialdehyde (MDA) production (D). Data were presented by the mean ± SEM from three independent experiments. *p < 0.05 compared with respective control group; **p < 0.05 compared with H₂O₂ only group; ns not significant compared with respective control group.

depolarization back to the same levels of control and GBE treated groups, suggesting the ability of GBE in protecting the integrity of mitochondria. In mammalian cells, mitochondrial depolarization causes release of pro-apoptotic factors from inter-membrane space (Sinha et al., 2013); we therefore investigated if the loss of viability in mouse cochlear NSCs upon H$_2$O$_2$ exposure to H$_2$O$_2$ was caused by increased apoptosis. Using flow cytometry for annexin V staining as apoptotic measurement, we found that exposure to H$_2$O$_2$ significantly induced apoptosis of mouse cochlear NSCs compared with control (Fig. 3B), and the addition of GBE in H$_2$O$_2$-exposed culture markedly reduced percentage of apoptotic cells, to a level similar to those of control and GBE alone treatment groups. Our aforementioned results clearly supported an anti-apoptotic function of GBE in protecting mouse cochlear NSCs from oxidative stress.

Ginkgo biloba extract inhibited intrinsic apoptotic pathway in mouse cochlear neural stem cells upon oxidative stress. So far we have established the anti-oxidative and anti-apoptotic effects of GBE in H$_2$O$_2$-induced oxidative stress in the in vitro cultured mouse cochlear NSCs, but the exact underlying molecular factors were still elusive. The mitochondrial apoptotic pathway is triggered by the combined action of the anti-apoptotic factor Bcl-2 and pro-apoptotic factor Bax, which induces the Caspase-3 dependent intrinsic apoptosis pathway (Bratton et al., 2001; Roy and Nicholson, 2000). We therefore examined the levels of the aforementioned factors of the intrinsic mitochondrial apoptotic pathway, using both RT-PCR and Western blot analyses. In mouse cochlear NSCs treated with H$_2$O$_2$ alone, mRNA level of anti-apoptotic factor Bcl-2 was significantly reduced (Fig. 4A), whereas mRNA level of pro-apoptotic factor Bax was markedly elevated (Fig. 4B). As expected, the combined changes in Bcl-2 and Bax expressions led to a more than three-fold upregulation of Caspase-3 (Fig. 4C), which is the master trigger factor of the intrinsic apoptosis pathway (Roy and Nicholson, 2000). Changes in protein expressions of all the aforementioned intrinsic apoptotic factors were confirmed to be the same as their respective mRNA levels (Fig. 4D). More importantly, addition of GBE into the H$_2$O$_2$-exposed culture was able to restore the expressions of these intrinsic apoptotic pathway factors to the same level of control (Fig. 4A, B, and C), clearly explaining its earlier observed anti-apoptotic function in this study.

**DISCUSSION**

In this study, we first isolated NSCs from mouse cochlea and treated them with H$_2$O$_2$ to establish an in vitro oxidative stress model. Exposure to H$_2$O$_2$ triggered strong oxidative stress in the culture, evident by excess production of ROS and mitochondrial depolarization, which triggered apoptosis through the intrinsic mitochondrial pathway. Apoptosis occurs through two different signaling pathways: the intrinsic and extrinsic pathways (Roy and Nicholson, 2000). The intrinsic (mitochondrial death) pathway of apoptosis is regulated by the combined actions of the pro-apoptotic and anti-apoptotic members of the Bcl-2 family proteins (Green and Reed, 1998). Among them, Bcl-2 is a major anti-apoptotic factor, which inhibits apoptosis by preventing the activation of inner mitochondrial permeability transition pore and release of pro-apoptotic mitochondrial contents including cytochrome c (Green and Reed, 1998). On the contrary, Bcl-2-like protein 4 (Bax) acts as pro-apoptotic factor, whose activation results in the release of pro-death proteins from the intermembrane space of the mitochondria into the cytosol. When released into the cytoplasm, cytochrome c recruits Caspase-9 which in turn induces Caspase-3 dependent apoptosis (Bratton et al., 2001).

Treatment with GBE in the H$_2$O$_2$-exposed cochlear NSC culture significantly rescued the loss in cell...
viability. Further investigation revealed that this beneficial role of GBE was mediated by its anti-oxidative functions, in that GBE could greatly attenuate oxidative stress by repressing ROS production and mitochondrial depolarization. Accumulation of ROS in the cells leads to cellular oxidative stress and is considered one major initiator of the intrinsic apoptosis pathway (Sinha et al., 2013). Therefore, we speculate the earlier observed anti-oxidative effect of GBE subsequently contributed to its anti-apoptotic effect in our current study, where GBE reversed the changes in the intrinsic apoptosis pathway factors by antagonizing oxidative stress caused by H₂O₂ challenge.

Our data indicated GBE at low and moderate concentrations (25 and 50 mg/L) increased cell viability, however at higher concentration (100 mg/L), GBE on the contrary inhibited cell viability. Similar biphasic effect of GBE was also reported, where low concentration of GBE was found to aggravate hypoxia/serum deprivation induced apoptosis, whereas high concentration significantly prevented BMSC apoptosis (Li et al., 2011). In fact, pro-apoptotic effect of GBE was observed in several in vitro cell models, including embryonic stem cells (Chan, 2006), effector T cells (Kotakadi et al., 2008), and tumor cells (Kim et al., 2005). We speculate these selective and divergent effects of GBE on different types of cells are likely due to variations in cell types and dosages.

Another intriguing observation among our data is that, although treatments with GBE alone at low and moderate concentration was consistently shown to be promotional to cell viability, the same dosage does not cause further significant reduction in oxidative stress or subsequent apoptosis, compared with media control without GBE or H₂O₂. It suggested that the promotional effect on mouse cochlear NSC viability (Fig. 1B) was only partially dependent on the anti-oxidative and anti-apoptotic functions of GBE. Indeed, the beneficial effects of GBE during oxidative stress seem to be diverse. It attenuates oxidative DNA damage induced by intermittent high glucose in human umbilical vein endothelial cells (He et al., 2014). Preconditioning with GBE was also reported to provide neuroprotection through heme oxygenase 1 and collapsin response mediator protein 2 pathway in mice (Nada and Shah, 2012). In the central nervous system, administration of GBE exhibited a variety of beneficial effects (Maclennan et al., 2002). It was found to enhance dopaminergic neurotransmission by inhibiting norepinephrine uptake, thereby improving cognition and attention in a mouse model (Fehske et al., 2009). GBE protects against neurotoxicity in mouse brain in an animal model of Parkinson’s disease by inhibiting apoptosis (Rojas et al., 2009). Moreover in a double-blind, randomized placebo-controlled clinical study, GBE improved mental/mnestic performance of human patients (Grassel, 1992). Underlying molecular mechanisms for all the aforementioned beneficial effects of GBE are not fully understood, but data from our current and other studies suggests different effects of GBE are likely to synergize in exerting its protective functions.

Figure 4. Attenuation of H₂O₂-induced apoptosis in mouse cochlear neural stem cells by Ginkgo biloba extract (GBE) was mediated by the intrinsic mitochondrial apoptotic pathway. Mouse cochlear neural stem cells were treated with medium only (control), 0.25 μM H₂O₂ only, 50 mg/L GBE only, or a combination of 0.25 μM H₂O₂ and 50 mg/L GBE for 24 h, followed by examination of mRNA levels of Bcl-2 (A), Bax (B), and Caspase-3 (C) by RT-PCR, as well as their protein levels (D) by Western blot analysis. Data were presented by the mean ± SEM from three independent experiments. *p < 0.05 compared with respective control group; #p < 0.05 compared with H₂O₂ only group; ns not significant compared with respective control group.
CONCLUSION
In summary, our study reveals that GBE can significantly attenuate hydrogen peroxide-induced oxidative stress and thereby enhance the viability of mouse cochlear NSCs in vitro. GBE also prevents mitochondrial depolarization, as well as the apoptosis that followed, therefore explaining its protective role on NSC viability. Furthermore, this anti-apoptotic role of GBE was found to be mediated by reversing the hydrogen peroxide-triggered changes in key intrinsic apoptosis pathway factors, such as Bcl-2, Bax, and Caspase-3. Our data provided the first report on the beneficial role of GBE in protecting mammalian cochlear NSCs from oxidative stress, therefore strongly supports the potential therapeutic use of GBE in preventing oxidative stress-related hearing loss.

Conflict of Interest
The authors declare no conflict of interest.

REFERENCES