Pigment epithelium-derived factor mitigates inflammation and oxidative stress in retinal pericytes exposed to oxidized low-density lipoprotein

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Abstract

Oxidized and/or glycated low-density lipoprotein (LDL) may mediate capillary injury in diabetic retinopathy. The mechanisms may involve pro-inflammatory and pro-oxidant effects on retinal capillary pericytes. In this study, these effects, and the protective effects of pigment epithelium-derived factor (PEDF), were defined in a primary human pericyte model. Human retinal pericytes were exposed to 100 μg/ml native LDL (N-LDL) or heavily oxidized glycated LDL (HOG-LDL) with or without PEDF at 10–160 nM for 24 h. To assess pro-inflammatory effects, monocyte chemotactic protein-1 (MCP-1) secretion was measured by ELISA, and nuclear factor-κB (NF-κB) activation was detected by immunocytochemistry. Oxidative stress was determined by measuring intracellular reactive oxygen species (ROS), peroxynitrite (ONOO⁻) formation, inducible nitric oxide synthase (iNOS) expression, and nitric oxide (NO) production. The results showed that MCP-1 was significantly increased by HOG-LDL, and the effect was attenuated by PEDF in a dose-dependent manner. PEDF also attenuated the HOG-LDL-induced NF-κB activation, suggesting that the inhibitory effect of PEDF on MCP-1 was at least partially through the blockade of NF-κB activation. Further studies demonstrated that HOG-LDL, but not N-LDL, significantly increased ONOO⁻ formation, NO production, and iNOS expression. These changes were also alleviated by PEDF. Moreover, PEDF significantly ameliorated HOG-LDL-induced ROS generation through up-regulation of superoxide dismutase 1 expression. Taken together, these results demonstrate pro-inflammatory and pro-oxidant effects of HOG-LDL on retinal pericytes, which were effectively ameliorated by PEDF. Suppressing MCP-1 production and thus inhibiting macrophage recruitment may represent a new mechanism for the salutary effect of PEDF in diabetic retinopathy and warrants more studies in future.

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Introduction

Diabetic retinopathy is a common microvascular complication of diabetes and the most frequent cause of vision loss in diabetic patients (Fong et al. 2003). In recent years, accumulating evidence suggests that inflammation is a key event in the pathogenesis of diabetic vascular complications (Abu El-Asrar et al. 1997, Mitamura et al. 2001, Hernández et al. 2005). In early stages of diabetic retinopathy, expression of pro-inflammatory cytokines, adhesion molecules, and chemokines is significantly up-regulated in the retina, in parallel with breakdown of blood–retina barrier and increased vascular permeability (Joussen et al. 2004, Zhang et al. 2006). Moreover, enhanced chemokine secretion into the retina and vitreous triggers recruitment and accumulation of monocyte/macrophage from blood to retina, contributing significantly to endothelial cell damage, capillary occlusion, non-perfusion, and neovascularization in the retina in diabetic retinopathy (Schröder et al. 1991, Tashimo et al. 2004).

Monocyte chemoattractant factor-1 (MCP-1) is a 14 kDa glycoprotein, commonly expressed in all vascular cells involved in inflammatory processes (Hernández et al. 2005). MCP-1 belongs to the CC chemokine family and is a potent chemoattractant for monocytes. The critical role of MCP-1 in diabetic vascular complications has been intensively studied and established in cardiovascular (macrovascular) diseases in diabetes (Sonoki et al. 2002, Renier et al. 2003). In diabetic retinopathy, MCP-1 levels in the vitreous are significantly elevated in patients with proliferative diabetic retinopathy (Abu El-Asrar et al. 1997, Mitamura et al. 2001, Hernández et al. 2005). In streptozotocin (STZ)-induced diabetic animals, expression of MCP-1 is also up-regulated in the retina and correlates with increased retinal vascular permeability (Zhang et al. 2006). In oxygen-induced ischemic retinopathy, a commonly accepted model for proliferative diabetic retinopathy, mRNA and protein levels of MCP-1 are increased in hypoxic inner retina at 3 and 12 h respectively after ischemia.
Pigment epithelium-derived factor (PEDF) is a major endogenous angiogenic inhibitor in the retina (Dawson et al. 1999). Decreased PEDF levels in the retina have been shown to be implicated in the pathogenesis of diabetic macular edema and proliferative diabetic retinopathy (Gao et al. 2002, Zhang et al. 2006). Previously, we reported that PEDF is a potent anti-inflammatory factor, inhibiting expression of pro-inflammatory factors, i.e. vascular endothelial growth factor (VEGF), tumor necrosis factor-α (TNF-α), and intercellular adhesion molecule-1 (ICAM-1), in retinal vascular endothelial cells (Zhang et al. 2006). Administration of PEDF into the vitreous significantly reduces retinal vascular leakage in diabetic animals (Zhang et al. 2006). Recent studies demonstrate that PEDF inhibits lipopolysaccharide-induced macrophage activation and induces macrophage apoptosis, suggesting a potential role of PEDF in ameliorating macrophage-implicated inflammatory events in diabetic retinopathy (Zamiri et al. 2006, Ho et al. 2008).

Oxidized low-density lipoprotein (LDL)-mediated inflammation and vascular damage are recognized as central to the pathogenesis of atherosclerosis and to the accelerated atherosclerosis of diabetes (Sonoki et al. 2002, Renier et al. 2003). An important mechanism for the deleterious effect of oxidized LDL on vascular function is to induce MCP-1 production and monocyte/macrophage recruitment (Sonoki et al. 2002, Renier et al. 2003). In diabetic retina, the disrupted blood–retinal barrier and increased vascular permeability result in extravasation and accumulation of LDL in the perivascular space, where it undergoes additional glycation and extensive oxidation. In a recent immunohistochemistry study, we demonstrated the presence of apoB (the apolipoprotein of LDL) and oxidized LDL in human retina from diabetic subjects in amounts increasing with the severity of diabetic retinopathy (Wu et al. 2008). By contrast, neither apoB nor oxidized LDL was detectable in normal retina. Our previous studies demonstrate that heavily oxidized glycated LDL (HOG-LDL; i.e., normal, pooled human LDL that has been modified in vitro first by glycation, then by oxidation to simulate conditions present in diabetes) induces apoptosis in retinal pericytes (Lyons et al. 2000). Moreover, HOG-LDL decreases the expression of tissue inhibitor of metalloproteinase-3 in retinal pericytes (Barth et al. 2007). Here, we hypothesize that HOG-LDL is a potent inducer of MCP-1 generation and oxidative stress in the retina, and that PEDF, an endogenous anti-inflammatory factor, suppresses HOG-LDL-induced MCP-1 production and thus inhibits monocyte/macrophage recruitment in diabetic retina. In the present study, we studied the effect of PEDF on HOG-LDL-induced MCP-1 production in retinal pericytes and explored the underlying mechanisms on nuclear factor-κB (NF-κB) pathway and oxidative stress.

Materials and methods

Materials

Dulbecco’s Modification of Eagles Medium (DMEM with 5 mmol/l d-glucose), hydrocortisone, human fibroblast growth factor B (hFGF-B), VEGF, R3-IGF-1, ascorbic acid, human epidermal growth factor (hEGF), and gentamicin sulfate and amphotericin-B (GA-1000) were purchased from Clonetics, Inc. (Walkersville, MD, USA). Fetal bovine serum (FBS) and antibiotic/mycotic solution were purchased from Gibco. Native LDL (N-LDL) was isolated from human plasma and HOG-LDL was prepared from N-LDL; both were characterized as described previously (Song et al. 2005). Human MCP-1 ELISA kit was purchased from Chemicon Inc. (Temecula, CA, USA). NF-κB activation assay kit was purchased from Cellomics Inc. (Pittsburgh, PA, USA). 5-(and-6)-Carboxy-2',7'-dichlorodihydrofluorescein (DCF) diacetate (carboxy-H2DCFDA) and Greiss reaction kit were purchased from Molecular Probe, Invitrogen Corporation. Anti-3-nitrotyrosine (3-NT) antibody was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-inducible nitric oxide synthase (iNOS) antibody was obtained from BD Biosciences (San Jose, CA, USA). Anti-superoxide dismutase 1 (SOD1) and anti-β-actin antibodies were purchased from Abcam, Inc. (Cambridge, MA, USA).

Cell culture

Primary human retinal pericytes were purchased from Clonetics, Inc. The cells were grown in medium containing 5% FBS, 0.04% hydrocortisone, 0.4% hFGF-B, 0.1% VEGF, 0.1% R3-IGF-1, 0.1% ascorbic acid, 0.1% hEGF, and 0.1% GA-1000 at 37°C in a 5% CO2 and 95% air incubator. After reaching 85% of confluence, the cells were treated in low-serum medium (containing 0.5% serum) for 24 h to obtain quiescence, and then exposed to the desired treatment in triplicate. The cells in passages 3–6 were used for experiments.

Western blot analysis of 3-NT, iNOS, and SOD1 in pericytes

Western blot analysis was performed as described previously (Zhang et al. 2004). Briefly, the cells were harvested and lysed in radioimmunoprecipitation assay.
(RIPA) lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% NP40, 0.25% Na-deoxycholate, 1 mM phenylmethylsulphonyl fluoride, protease inhibitor, and phosphatase inhibitor. Protein concentration was measured with the Bio-Rad DC protein assay kit. Fifty microgram of protein from total cell lysate were separately blotted with anti-3-NT (1:1000), anti-iNOS (1:1000), and anti-SOD1 (1:2000). The same membrane was stripped and reblotted with an anti-β-actin antibody (1:4000) as control.

Quantification of MCP-1 secretion in pericytes

MCP-1 secreted into the medium was determined using a sandwich enzyme immunoassay following the manufacturer’s instruction. Briefly, the cell culture supernatant was collected after treatment and centrifuged to remove any visible particulate material. Standards or sample (100 μl) were added to the antibody pre-coated microtiter plate. Simultaneously, 25 μl rabbit anti-Human MCP-1 polyclonal antibody were dispensed into each well. The mixture was incubated at room temperature for 3 h. After four washings, 50 μl goat anti-rabbit conjugated alkaline phosphatase were added, followed by incubation for 45 min. The substrate was then added and incubated for 5 min, the plate was read immediately at 490 nm in a Victor microplate reader. The MCP-1 concentration was calculated according to the standard curve and normalized by cell numbers.

NF-κB activation assay

NF-κB activation was evaluated by an NF-κB activation assay kit based on immunofluorescein method. Briefly, cultured pericytes were seeded and grown to 80% confluence on four-chamber slides (Nalge Nunc International Corp., Naperville, IL, USA). After quiescence for 24 h, the cells were treated with N-LDL or HOG-LDL at 100 μg/ml in the presence or absence of PEDF for 24 h. After fixation with 3%–7% formaldehyde for 10 min and permeabilization for 5 min, the cells were incubated with primary and secondary antibodies for 1 h. The nuclei were counterstained with Hoechst dye. After extensive washing, the slides were visualized and photographed under a fluorescent microscope (Olympus, Hamburg, Germany).

Detection of intracellular reactive oxygen species (ROS) generation

The cells were seeded in a 96-well plate and grown to 85% confluence. After quiescence for 24 h, the cells were exposed to 100 μg/ml N-LDL or HOG-LDL with or without PEDF for 24 h. The generation of intracellular ROS was detected by DCF method using carboxy-H2DCFDA, a cell-permeable indicator for ROS (Obrosova et al. 2005). Briefly, the cells were gently washed with PBS and incubated with 2 μM carboxy-H2DCFDA in phenol red-free medium at 37 °C for 20 min. The medium was discarded and the cells were washed with PBS. Fluorescence was measured by a fluorescence microplate reader with an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

Measurement of nitric oxide (NO) production in pericytes

NO production was evaluated by measuring the accumulation of nitrates, a stable oxidative end product of NO metabolism, in the supernatant of cultured pericytes using the Greiss reagent kit following the manufacturer’s instruction. Briefly, the samples were incubated with 100 μIU/ml nitrate reductase and 20 μg/ml NADPH to convert nitrates to nitrites for analysis. Then, 150 μl sample was added to 20 μl premixed Greiss reagent (containing sulfanilamide and N-[1-naphthyl]ethylenediamine) and 130 μl deionized water in a 96-well microplate and incubated at room temperature for 30 min. The optical density was measured with a Victor microplate reader at a wavelength of 546 nm. Nitrite concentrations in the supernatants were calculated according to the standard curve.

Statistical analysis

Data were calculated and expressed as group means ± s.d. Statistical analyses were performed using Student’s t-test, ANOVA, and Bonferroni’s multiple comparison test. Statistical differences were considered significant at a P value of less than 0.05.

Results

HOG-LDL induces MCP-1 secretion in retinal pericytes, and PEDF inhibits this effect

Oxidized LDL is a major inducer of MCP-1 expression in vascular smooth muscle cells and plays a central role in diabetic macrovascular complications (Mampulu & Renier 2001). However, the effect of oxidized LDL on diabetic retinopathy is under-investigated. In the present study, we determined MCP-1 secretion induced by HOG-LDL in retinal pericytes. Cultured primary human retinal pericytes were exposed to 100 μg/ml N-LDL or 100 μg/ml HOG-LDL for 24 h. MCP-1 secreted into the medium was measured by ELISA. The results showed that MCP-1 secretion from the pericytes exposed to HOG-LDL was significantly elevated when compared with those exposed to N-LDL or normal control (Fig. 1, P<0.01, ANOVA). No significant difference was observed in MCP-1 levels between the cells exposed to N-LDL and normal controls.
These results indicated that the effect of NF-κB was activated by HOG-LDL. The nuclear translocation distributed in cytoplasm in control cells (Fig. 2A and B). The results showed that the signal of NF-κB from cytoplasm to nuclei using immunocytochemistry. After incubation with HOG-LDL (Fig. 2E and F) but not N-LDL (Fig. 2C and D) for 24 h, NF-κB translocated from cytoplasm to nuclei (Fig. 2C–F), indicating that NF-κB was activated by HOG-LDL. The nuclear translocation of NF-κB was almost completely blocked by PEDF (Fig. 2G and H). These results indicated that the effect of PEDF on reducing MCP-1 production was at least partially through inhibition of NF-κB activation.

**HOG-LDL-induced peroxynitrite formation in retinal pericytes; PEDF inhibits this effect**

Oxidative stress has been recognized as a critical mediator for inflammation and NF-κB activation induced by oxidized LDL (Renier et al. 2003). Peroxynitrite (ONOO⁻) is a potent oxidant, which initiates both nitrosative and oxidative reactions affecting cellular proteins, lipids, and DNA, and resulting in an increase in oxidative stress and a decrease in antioxidant defenses (Zou et al. 2002). Scavenging of ONOO⁻ effectively diminishes diabetes-induced retinal vascular leakage, suggesting a crucial role of ONOO⁻ in diabetic retinopathy (El-Remessy et al. 2003). We hypothesize that PEDF inhibits HOG-LDL-induced NF-κB activation via suppressing oxidant generation in retinal pericytes. To test this hypothesis, we determined the formation of peroxynitrite in cultured retinal pericytes exposed to HOG-LDL in the presence or absence of PEDF. Peroxynitrite was detected by western blot analysis of 3-nitrotyrosine (3-NT)-positive proteins, which has been commonly accepted as a ‘footprint’ of ONOO⁻ in cell culture (Zou et al. 2002, Obrosova et al. 2005). The results showed that 100 μg/ml HOG-LDL induced an increase in 3-NT levels (Fig. 3). N-LDL at the same concentration did not increase 3-NT formation. The addition of PEDF (160 nM) partially inhibits HOG-LDL-induced 3-NT formation but did not restore to control levels (Fig. 3).

**NF-κB activation in retinal pericytes was induced by oxidized LDL, and the effect was inhibited by PEDF**

NF-κB activation is an important transcriptional factor regulating the expression of a variety of pro-inflammatory cytokines and adhesion molecules, including MCP-1 (Sonoki et al. 2002, Renier et al. 2003). Moreover, the previous studies suggest that NF-κB activation is an essential step in the overproduction of MCP-1 induced by oxidized LDL or glycated and oxidized LDL in vascular cells (Sonoki et al. 2002, Renier et al. 2003). Thus, we next determined whether PEDF reduces HOG-LDL-induced MCP-1 secretion through the inhibition of NF-κB activation. Cultured human retinal pericytes were exposed to 100 μg/ml N-LDL or HOG-LDL in the presence or absence of 160 nM PEDF for 24 h. NF-κB activation was analyzed by the translocation of NF-κB from cytoplasm to nuclei using immunocytochemistry. The results showed that the signal of NF-κB was diffusely distributed in cytoplasm in control cells (Fig. 2A and B). After incubation with HOG-LDL (Fig. 2E and F) but not N-LDL (Fig. 2C and D) for 24 h, NF-κB translocated from cytoplasm to nuclei (Fig. 2C–F), indicating that NF-κB was activated by HOG-LDL. The nuclear translocation of NF-κB was almost completely blocked by PEDF (Fig. 2G and H). These results indicated that the effect of PEDF on reducing MCP-1 production was at least partially through inhibition of NF-κB activation.

**HOG-LDL-induced iNOS expression and NO production in retinal pericytes, and effects were blocked by PEDF**

ONOO⁻ is formed via the reaction of O₂⁻ with NO (Zou et al. 2002). The overproduction of NO not only provides an essential precursor for ONOO⁻ generation but also contributes to pericyte loss in diabetic retinopathy (Miller et al. 2006). In this study, we determine the effects of HOG-LDL on NO production and iNOS expression in retinal pericytes in the presence and absence of PEDF. The results showed that the levels of iNOS expression (Fig. 4A) and NO production (Fig. 4B) were very low in control cells. HOG-LDL, but not N-LDL, significantly increased iNOS expression and correspondent NO production, which were significantly reduced by 160 nM PEDF (Fig. 4).

**PEDF-reduced ROS production through up-regulation of SOD1 expression in retinal pericytes**

We determined whether HOG-LDL-induced ROS generation in pericytes, and the effects of PEDF. Cultured human retinal pericytes were exposed to 100 μg/ml N-LDL or HOG-LDL with or without different concentrations of PEDF at 10, 40, and 160 nM for 24 h. MCP-1 secreted into the medium was measured by ELISA without different concentrations of PEDF at 10, 40, and 160 nM for 24 h. The values statistically different from untreated control and that treated with N-LDL are indicated by *P<0.05, **P<0.01.

**Figure 1** Blockade of oxidized LDL-induced MCP-1 secretion by PEDF in retinal pericytes. Cultured human retinal pericytes were exposed to 100 μg/ml N-LDL or 100 μg/ml HOG-LDL with or without different concentrations of PEDF at 10, 40, and 160 nM for 24 h. MCP-1 secreted into the medium was measured by ELISA (mean ± s.d., n=3). The values statistically different from untreated control and that treated with N-LDL are indicated by #P<0.05, from HOG-LDL as *P<0.05, **P<0.01.
retinal pericytes were exposed to 100 μg/ml N-LDL or HOG-LDL in the presence or absence of 160 nM PEDF for 24 h. The results showed that HOG-LDL significantly increased, and the addition of PEDF significantly decreased, ROS generation (Fig. 5A). To further elucidate how PEDF inhibits ROS generation, we determined the effect of PEDF on expression of SOD1 in retinal pericytes.
Exposed to HOG-LDL in the presence or absence of PEDF at 10, 40, and 160 nM. The results showed that HOG-LDL treatment for 24 h drastically decreased SOD1 expression in retinal pericytes, and that this was restored by PEDF in a dose-dependent manner (Fig. 5B).

Discussion

In this study, we demonstrated for the first time that oxidized, glycated LDL increased ROS generation, nitric oxide production, and peroxynitrite formation, which subsequently activated NF-κB pathway, leading to overproduction of MCP-1 in retinal pericytes. PEDF ameliorates oxidative stress and suppresses NF-κB activation and thus, reduces MCP-1 production. The inhibitory effect of PEDF on MCP-1 production contributes to its salutary role in reducing vascular leakage and inhibiting neovascularization in diabetic retinopathy.

Pericytes and endothelial cells are the two major cell components of retinal blood capillaries. Pericytes are traditionally believed not to be in direct contact with the circulation because of the inner blood–retinal barrier. However, capillary leakage occurs early in diabetic retinopathy, and extravasation and entrapment of LDL in the sub-endothelial space may allow for continuing glycation and oxidation (processes already started in the circulation), leading to local accumulation of various concentrations of severely modified particles (Qaum et al. 2001, Zhang et al. 2005, 2006). In a recent study, we used immunostaining to show that both apoB and oxidized LDL are present in human retina in the presence of diabetes, in amounts proportional to the severity of diabetic retinopathy, but are entirely absent in non-diabetic retina (Wu et al. 2008). Consistent with this, Qaum et al. (2001) demonstrated that in rats, after induction of diabetes, the retinal vasculature is permeable to microspheres as large as 100 nm in diameter, whereas by comparison, LDL particles are much smaller with a diameter of ~ 20 nm. Furthermore, accumulation of LDL in the vessel wall may be particularly high, as evidenced by the finding of a twofold or more higher level of LDL within the intima of normal human aortas than in plasma (Smith & Staples 1982), suggesting retention of the particles. Also in human arteries, the concentration of oxidized LDL in atherosclerotic plaques was found to be 70 times higher than in plasma (Nishi et al. 2002). Given the complexity of this situation, we chose the LDL concentration of 100 μg protein/ml for our studies. We believe that this reflects a conservative estimate of conditions in the retina in vivo in the presence of diabetes, since the concentration range of apoB in normal plasma is 700–1200 μg/ml (Nishi et al. 2002).
We reported recently that PEDF is a potent endogenous anti-inflammatory factor (Zhang et al. 2006). Decreased intracellular PEDF levels have been observed in patients with diabetic retinopathy and in animal models of type 1 diabetes and spontaneous uveitis (Boehm et al. 2003, Zhang et al. 2006, Hauck et al. 2007). Administration of PEDF into the vitreous significantly inhibits leukostasis and reduces vascular permeability in the retina in STZ-induced diabetic rats, suggesting a potential role of PEDF on leukocyte/macrophage death, PEDF is likely able to ameliorate monocyte/macrophage recruitment into the retina by suppressing MCP-1 production. Recent studies showed that overproduction of MCP-1 in the retina and recruitment of hematogenous macrophages into the retina plays a causative role in retinal neovascularization in ischemia-induced retinopathy (Davies et al. 2006, Shen et al. 2007). Thus, future studies are warranted to determine whether inhibition of monocyte/macrophage recruitment and activation is a novel mechanism responsible for the anti-angiogenesis effect of PEDF in diabetic retinopathy.

NF-κB is activated by a variety of stimuli and plays a critical role in the regulation of multiple cytokines, such as ICAM-1 and MCP-1 (Kowluru & Koppolu 2002, Sonoki et al. 2002, Renier et al. 2003); although other transcription factors, e.g. activator protein-1 have been shown to be partially responsible for MCP-1 regulation (Chen et al. 2004). Previous studies have demonstrated that oxidized LDL and glycoxidized LDL are potent inducers of NF-κB activation in vascular cells and monocyte/macrophages (Do carmo et al. 1998, Sonoki et al. 2002). Moreover, the results from electrophoretic mobility shift assay and chromatin immunoprecipitation assays showed that oxidized glycated LDL and oxidized LDL induced MCP-1 expression via NF-κB activation. In the present study, we demonstrated that HOG-LDL caused translocation of NF-κB from cytoplasm to nucleus, which was blocked by PEDF. Although nuclear translocation of NF-κB does not represent direct evidence of NF-κB activation, translocation of NF-κB to the nucleus is an essential step for subsequent DNA binding and transcription activation. Inhibition of NF-κB nuclear translocation by PEDF would thus suppress subsequent NF-κB DNA binding and activation. Interestingly, previous studies showed that activation of NF-κB in neurons is responsible for the neuroprotective activity of PEDF (Yabe et al. 2001). In our present study, we did not observe a protective effect of PEDF on HOG-LDL-induced apoptosis of pericytes nor activation of NF-κB by...
PEDF (data not shown), suggesting that the effect of PEDF on NF-$kappaB$ activation is cell-type specific. Taken together, the present study demonstrates that PEDF alleviated HOG-LDL-induced MCP-1 overproduction through blockade of NF-$kappaB$ activation. The inhibitory effect of PEDF on oxidative stress was attributed to its anti-inflammatory activity on macrophage recruitment, and thus may contribute to the antiangiogenesis effect of PEDF in diabetic retinopathy.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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