Original Paper

Dihydroartemisinin Accentuates the Anti-Tumor Effects of Photodynamic Therapy via Inactivation of NF- κ B in Eca109 and Ec9706 Esophageal Cancer Cells

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Key Words

Apoptosis • Dihydroartemisinin • Esophageal cancer • NF- κ B • Photodynamic therapy

Abstract

Background: Photodynamic therapy (PDT) is a new treatment for esophageal cancer which has been shown to be effective in the elimination of tumor. However, PDT could induce the activation of nuclear factor-kappa B (NF- κ B) in many photosensitizers based PDT, which plays a negative role in PDT. In addition, our previous results have shown that dihydroartemisinin (DHA), which was the most potent one of artemisinin derivatives, has anticancer activity in esophageal cancer cells. **Methods:** Cell viability was determined by MTT analysis, and apoptosis was evaluated by flow cytometry. Nuclear extract was obtained for determining NF- κ B DNA-binding activity, while total protein extract obtained for downstream gene expression by western blot. **Results:** We demonstrated DHA enhanced PDT-induced growth inhibition and apoptosis in both human esophageal cancer cell lines Eca109 and Ec9706 in vitro. The mechanism was at least partially due to DHA deactivated PDT-induced NF- κ B activation, so as to decrease tremendously the expression of its target gene Bcl-2. **Conclusion:** Our results demonstrate that DHA augments PDT-induced growth inhibition and apoptosis in esophageal cancer cells, and that inactivation of NF- κ B activity is a potential mechanism by which DHA sensitizes esophageal cancer cells to PDT-induced growth inhibition and apoptosis.

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Introduction

Esophageal cancer is the eighth-most common cause of malignancy worldwide, and it ranks as the fourth leading cause of all cancer deaths in China [1, 2]. Despite extensive research and the use of surgical resection, chemotherapy and radiotherapy, the 5-year survival rate is only 15% [3]. This low survival rate could be due to the lack of early diagnosis, invasion and metastases of the tumor and high resistance to chemotherapy and radiation. Therefore, it is necessary to develop a new strategy to prolong the survival of patients with esophageal cancer.

Photodynamic therapy (PDT) is a new treatment that has been shown to be effective in the elimination of esophageal cancer [4-6]. PDT uses a photosensitizer and visible light of appropriate wavelength, resulting in the production of reactive oxygen species that destroy tumors and produce photochemical effects in the target area [7].

However, PDT could induce the activation of nuclear factor-kappa B (NF- κ B) [8-10]. Several studies have indicated that NF- κ B activation plays a negative role in PDT. Matroule et al. found that the HCT-116 cell line, which expresses a dominant-negative mutant of inhibitor- $\kappa\alpha$, was highly sensitive to apoptosis and indicated that NF- κ B activation was involved in an anti-apoptotic response to aminopyropheophorbide (APP)-mediated photosensitization [11]. Furthermore, it has been demonstrated that inhibition of NF- κ B improves glioblastoma cell death in response to 5-Aminolevulinic acid (5-ALA)-mediated PDT [12]. In particular, NF- κ B is known to activate the transcription of anti-apoptotic factors, such as cIAPs, and anti-apoptotic members of the Bcl2 family, such as A1/Bfl1 and Bcl-XL [13]. Therefore, inhibiting NF- κ B activation may be a novel strategy to enhance the activity of PDT.

Dihydroartemisinin (DHA), which is the most potent of the artemisinin derivatives, exhibits anticancer activity in pancreatic cancer [14], colorectal cancer [15], osteosarcoma and others [16]. In a previous study, we demonstrated that DHA might be a novel agent against esophageal cancer [17]. Furthermore, DHA inactivated NF- κ B and potentiated the anti-tumor effect of gemcitabine on pancreatic cancer [14]. It has also been demonstrated that DHA inhibited NF- κ B activity in osteosarcoma cells [16].

Taken together, DHA exhibits anticancer activity and inhibits NF- κ B activity. We hypothesized that DHA could enhance the anti-tumor effect of PDT on esophageal cancer cells via the inhibition of NF- κ B. Cell proliferation, apoptosis, NF- κ B activity and the expression of NF- κ B-regulated gene were all investigated in this study.

Materials and Methods

Cells

The human esophageal cancer cell lines Eca109 and Ec9706 (Laboratory of Medical Genetics, Department of Biology, Harbin Medical University) were grown in RPMI 1640 medium containing 10% heated inactivated fetal bovine serum (FBS) and 100 units/ml penicillin/streptomycin. Cells were maintained in a humidified atmosphere of 5% CO₂ at 37°C.

Materials

DHA (Sigma-Aldrich, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma) and stored at -20°C. 5-ALA was reconstituted in RPMI 1640 medium and stored at -20°C. Annexin V-FITC and the ECL Plus system were purchased from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Amresco, USA) was dissolved to a final concentration of 5mg/mL in PBS. Antibodies against p65, Bax, caspase-3 and caspase-9 were purchased from Cell Signaling Technology (Beverly, MA, USA); and an antibody against Bcl-2 and horseradish peroxidase-conjugated secondary antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

PDT treatment

Eca109 and Ec9706 cells were incubated in 96- or 6-well, flat-bottomed microplates, and the supernatants were removed and replaced with 200 μ l of fresh, FBS-free medium. The cells were then

treated with 5-ALA (0.1–1 mmol/L) for 4 h. Prior to irradiation, the cells were washed three times with PBS and replaced with another 200 μ l of RPMI 1640 before illumination. Irradiation was carried out using a 630-nm wavelength Diomed 630 PDT system (Diomed Inc. United Kingdom) at respectively fluence rates of 25 W/cm² and 20 W/cm². After irradiation, the medium was replaced with RPMI 1640 containing 10% FBS and no or different concentrations of DHA.

Cell viability assay

Eca109 (4×10^3 cells/well) and Ec9706 (5×10^3 cells/well) cells were grown in 96-well plates and cultured overnight to allow for cell attachment. Eca109 and Ec9706 cells were treated with DHA (80 μ mol/L), PDT (25 and 20 J/cm², respectively) or their combination. After incubation for 24h, MTT (20 μ l) was added to each well and incubated for 4 h at 37°C. Formazan crystals were dissolved in 150 μ l of DMSO for 10 min with shaking. The absorbance was measured at 490 nm on a plate reader, and the experiment was repeated three times.

Cell apoptosis assay

Eca109 (2×10^5 cells/well) and Ec9706 (2×10^5 cells/well) cells were grown in 6-well plates and cultured overnight to allow for cell attachment. After the designated treatment 24h later, Eca109 and Ec9706 cells were washed three times with ice-cold PBS. The cells were then re-suspended in 195 μ l binding buffer, 5 μ l Annexin V-FITC and 10 μ l propidium iodide (PI) were added, and the cells were incubated for 20 min at room temperature in the dark. Before measurements, the cells were diluted with 190 μ l binding buffer and filtered, and single-cell suspensions were analyzed on a flow cytometer (Epics Altra II, Beckman Coulter, USA). The summation of early and late apoptotic cells was used to calculate the apoptosis rate. The experiments were repeated three times.

Transmission electron microscopy (TEM)

Eca109 (2×10^5 cells/well) and Ec9706 (2×10^5 cells/well) cells were grown in 6-well plates and cultured overnight to allow for cell attachment. After the designated treatment 24h later, the cells were harvested, washed and fixed with 2.5% glutaraldehyde (Sigma-Aldrich, G6257) containing 1% tannic acid overnight. After washing, the cell pellets were embedded in epon araldite, ultra-thin sections were observed using a Hitachi-h7650 electron microscope, and representative images were analyzed.

Western blot analysis

Eca109 (2×10^5 cells/well) and Ec9706 (2×10^5 cells/well) cells were grown in 6-well plates and cultured overnight to allow for cell attachment. After the designated treatment 24h later, whole-cell lysates were prepared using RIPA buffer (Beyotime Institute of Biotechnology, Jiangsu, China). The total proteins were centrifuged at 14000 r/min for 10 min at 4°C and then quantified using the Bradford assay. The protein contents of the cell homogenates were determined, and samples containing 30 μ g total protein were resolved on 12% polyacrylamide SDS gels and blotted onto polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk and incubated overnight at 4°C with primary antibodies. After 30 min of washing in wash buffer, the membranes were incubated with the appropriate horseradish-conjugated secondary antibody for 1 h at room temperature. The blots were visualized using a chemiluminescence detection kit (ECL-PLUS), and anti- β -actin was used to ensure equal loading.

Electrophoretic Mobility Shift Assay (EMSA)

Eca109 (2×10^5 cells/well) and Ec9706 (2×10^5 cells/well) cells were grown in 6-well plates and cultured overnight to allow for cell attachment. After the designated treatment 24h later, the cells were washed three times in cold PBS, scraped in cold PBS and centrifuged at 5000 r/min for 5 min. The nuclear protein was obtained using a nuclear extract kit (Thermo Scientific, USA), and protein concentrations were measured using the BCA protein assay (Thermo Scientific, USA). The binding reaction was performed using the EMSA/Gel-Shift kit (Thermo Scientific, USA), and DNA-protein complexes were resolved by 3h electrophoresis in native 6% polyacrylamide gels at 200V using 0.25 mol/L Na borate, 0.5 mmol/L EDTA and 0.25 mol/L Tris, pH 8.0. The gels were vacuum-dried and exposed to Fuji x-ray film at -80°C for 16 to 25h. The sequences of the probes used in this work were according to a previous study [18]: wild type NF- κ B probe, 5'-GGTTACAAGGGACTTTCGCTG-3', and mutated NF- κ B probe, 5'-GGTTAACAACCTCACTTTCGCTG-3'. The

Fig. 1. The viability of cells was assessed by calculating the viability index using the MTT method. (a) DHA inhibits the proliferation of human esophageal cancer cells in vitro. Two human esophageal cancer cell lines, Eca109 and Ec9706, were exposed to various concentrations of DHA (2.5–120 μmol/L) for 24 h. (b) PDT inhibits the proliferation of human esophageal cancer cells in vitro. After treatment with 5-ALA (0.1–1 mmol/L) for 4 h, PDT was carried out at a fluence rate of 25 W/cm² and 20 W/cm². Values represent the mean ± SD.

Fig. 2. The PDT effect on cell viability is potentiated by the addition of DHA. Eca109 and Ec9706 cells were treated with DHA (80 μmol/L), PDT (incubated with 5-ALA at 0.5 mmol/L for 4 h, 25 and 20 J/cm², respectively) or their combination, before the cell viability index was generated by MTT assay. ‘*’ marks statistical significance (p < 0.05), when compared with single treatment.

dried gels were visualized using a CoolImager (IMGR002), and radioactive bands were quantified using the Scion Image software.

Statistical analysis

All results were expressed as the mean values ± standard deviation, except for the results from the western blot assay. One-way ANOVA was used for statistical analysis. A value of less than 0.05 (* $P < 0.05$) was used for statistical significance.

Results

DHA reduced the viability of esophageal cancer cells

We previously reported the dose responses of DHA in Eca109 and Ec9706 cells for 48 and 72 h, respectively (data not shown) [17]. In the present study, we set out to investigate the dose responses of DHA in Eca109 and Ec9706 cells exposed to various concentrations of DHA (2.5–120 μmol/L) for 24 h. The effect of DHA on the viability of the two types of cells was detected using the MTT assay. As shown in Fig. 1a, DHA reduced the viability of the two types of cells in vitro in a dose-dependent manner when compared with untreated cells. These results suggested that DHA was cytotoxic to human esophageal cancer cells.

PDT inhibited the proliferation of esophageal cancer cells in vitro

Chen et al. suggested that 5-ALA-PDT significantly inhibited cell proliferation of Eca109 cells [5]. In our study, we set out to investigate if PDT could inhibit the proliferation of other

Fig. 3. The apoptotic index was determined by flow cytometry. Eca109 and Ec9706 cells were treated with DHA (80 $\mu\text{mol/L}$), PDT (incubated with 5-ALA at 0.5mmol/L for 4 h, 25 and 20 J/cm^2 , respectively) or their combination.

Fig. 4. The percentages of apoptotic cells. Eca109 and Ec9706 cells were treated with DHA (80 $\mu\text{mol/L}$), PDT (incubated with 5-ALA at 0.5mmol/L for 4 h, 25 and 20 J/cm^2 , respectively) or their combination. '*' marks statistical significance ($p < 0.05$), when compared with single treatment.

esophageal cancer cell line, Ec9706 cells. In accordance with their study, the results of our MTT assay revealed that PDT significantly inhibited the proliferation of human esophageal cancer cells. When Eca109 and Ec9706 cells were exposed to 25 J/cm^2 and 20 J/cm^2 , PDT increased the amount of cell damage as the 5-ALA concentration increased when compared with the control group (Fig. 1b).

DHA augmented PDT-induced growth inhibition in esophageal cancer cells

PDT could induce the activation of nuclear factor-kappa B (NF- κ B) in many photosensitizers based PDT, which played a negative role in PDT [8-10]. In addition, DHA could inhibit NF- κ B activity [14, 16]. Therefore, we hypothesized that DHA could enhance the anti-tumor effect of PDT on esophageal cancer cells, and we investigated cell viability using the MTT assay. Eca109 and Ec9706 cells were treated with DHA (80 $\mu\text{mol/L}$), PDT (25 and 20 J/cm^2 , respectively) or their combination. We found that single treatment with DHA or PDT caused a $37 \pm 5\%$ or $34 \pm 6\%$ reduction in viability in Eca109 cells and a $33 \pm 7\%$ or $34 \pm 6\%$ reduction in Ec9706 cells, respectively. However, when PDT was combined with DHA, the cell viability was reduced $59 \pm 6\%$ or $61 \pm 7\%$ in the cell lines, respectively (Fig. 2).

Fig. 5. Morphological changes of the cells by TEM observation. No obvious cell damage was observed in the control group. Apoptosis and necrosis occurred in the treatment group. (a) Control, untreated Eca109 cells; (c) Control, untreated Ec9706 cells; (b) Eca109 cells treated with PDT combined with DHA; and (d) Ec9706 cells treated with PDT combined with DHA.

DHA sensitized esophageal cancer cells to PDT-induced apoptosis

Several studies have demonstrated that DHA could induce apoptosis in pancreatic cancer, colorectal cancer, osteosarcoma and others [14-16]. Therefore, we sought to determine the role of apoptosis in the enhancement of the anti-tumor effect of PDT by DHA. Apoptotic changes were detected by flow cytometric analysis using AnnexinV-FITC and PI staining (Fig. 3). Eca109 and Ec9706 cells were treated with DHA (80 $\mu\text{mol/L}$), PDT (25 and 20 J/cm^2 , respectively), or their combination. As shown in Fig. 3 and 4, DHA increased the apoptotic rate from $7.7 \pm 1.2\%$ to $12.1 \pm 1.4\%$ in Eca109 cells and from $5.5 \pm 0.8\%$ to $12.7 \pm 1.7\%$ in Ec9706 cells, while single PDT increased the rate from $7.7 \pm 1.2\%$ to $11.2 \pm 1.4\%$ in the former and from $5.5 \pm 0.8\%$ to $12.3 \pm 2.4\%$ in the latter, all with statistical significance ($P < 0.05$). Combination of DHA and PDT produced significantly more pronounced apoptosis compared with single treatment in the respective cells ($20.9 \pm 1.9\%$ and $21.8 \pm 2.0\%$, both with $P < 0.05$).

Ultrastructural morphologic changes in human esophageal cancer cells

Our studies demonstrated that DHA could induce apoptosis in esophageal cancer cells [17]. To further confirm cell apoptosis, we examined the ultrastructural morphologic changes under TEM. In the control group, no obvious cell damage was observed in Eca109 (Fig. 5a) or Ec9706 cells (Fig. 5c). However, in the treatment group, some cells showed typical characteristics of apoptotic and necrotic cells, such as apoptotic bodies, nuclear condensation and broken cell membranes with nuclear lysis (Fig. 5b, d).

DHA abrogated PDT-induced NF- κ B activation and facilitated the down regulation of its downstream gene expression

Several studies have shown that PDT can induce the activation of NF- κ B in PDT, and that NF- κ B activation plays a negative role in PDT [8-10]. Furthermore, DHA can inhibit NF- κ B activity in pancreatic cancer and osteosarcoma cells [14, 16]. Therefore, we set out to investigate if DHA could enhance the anti-tumor effect of PDT on esophageal cancer cells via inhibition of NF- κ B. Eca109 and Ec9706 cells were treated with DHA (80 $\mu\text{mol/L}$), PDT (25 and 20 J/cm^2 , respectively), or their combination. Nuclear extract was then obtained to determine the NF- κ B DNA-binding activity using EMSA assays, while total protein extract was obtained to analyze downstream gene expression by western blotting. As shown in Fig. 6, Eca109 and Ec9706 cells constitutively expressed NF- κ B, and PDT alone obviously

Fig. 6. NF- κ B DNA-binding activity. Lanes represent: positive control (PC), control (C), DHA single treatment (D), PDT single treatment (P) and combined treatment (DP). (a) EMSA assay for NF- κ B DNA-binding activity on nuclear extracts. (b) Densitometric quantification of the data presented in the top panel. “*” $P < 0.05$, compared with PDT single treatment.

Fig. 7. NF- κ B-targeted gene and apoptosis-related protein expression. Lanes represent: control (C), DHA single treatment (D), PDT single treatment (P) and combined treatment (DP). Western blot analysis of the expression of p65, Bcl-2, Bax and procaspase-3 and 9 from cell homogenates, with β -actin as a protein loading control.

enhanced its DNA-binding activity when compared with the control in Eca109, but not in Ec9706 cells. However, in both cell types, DHA significantly abrogated the inducing effect of PDT on NF- κ B activation.

Furthermore, PDT up-regulated the expression of p65 compared with the control (Fig. 7), and we found that DHA down-regulated its expression when in combination with PDT. Moreover, as shown in Fig. 7, combined treatment down-regulated the expression of Bcl-2, a target of NF- κ B, and up-regulated Bax and increased caspase-3 and caspase-9 activation, all of which increased apoptosis in both cell types.

Discussion

Patients with esophageal cancer are often diagnosed at the advanced stage and with esophageal obstruction, which seriously affects the life quality of patients. Many patients often lose the optimal chance for surgery or even the chance for chemotherapy or radiotherapy. An effective medical treatment involves relieving the esophageal obstruction by PDT, which induces localized tumor destruction via the photochemical generation of cytotoxic singlet oxygens. 5-ALA is a physiological compound and the precursor of protoporphyrin IX (PPIX), which is a strong photosensitizer that is used as an emerging treatment strategy for various cancers. The sensitizer is harmless unless it is metabolized into PPIX and activated by light of the appropriate wavelength. In addition, 5-ALA-PDT has been investigated in clinical and experimental studies on esophageal cancer [5, 6]. In the present study, we found that 5-ALA-PDT could induce cell apoptosis and reduce cell viability in esophageal cancer cells in vitro. In accordance with our study, Chen et al. suggested that 5-ALA-PDT significantly induced esophageal cancer cell apoptosis and inhibited cell proliferation [5].

NF- κ B is a major transcription factor that regulates various cell processes, such as embryonic development, immunity, apoptosis, angiogenesis and proliferation [19]. The NF- κ B family is composed of five members (RelA or p65, RelB, c-Rel, p50 and p52). In resting cells, NF- κ B is sequestered in the cytoplasm through the interaction with an inhibitor of the I κ B family. However, it can be activated and transported into the nucleus by a large number of different stimuli, such as cytokines, bacterial or viral compounds, oxidative stress, DNA damage and others. Once in the nucleus, it can activate the transcription of its target genes.

It was demonstrated that NF- κ B was activated in the photodynamic response of HL-60 cells to verteporfin [7]. Matroule et al. suggested that PPME photosensitization activated NF- κ B in colon cancer cells [8], and in a study of the hypericin-mediated photocytotoxic effects on HT-29 adenocarcinoma cells, an NF- κ B activity assay confirmed the activation of this early response pathway [9]. Mladen Korblick has found the phenomenon of activation of NF- κ B in photofrin-mediated PDT in a mouse SCCVII tumor model [10]. Consistent with the above-mentioned studies, we found that 5-ALA-PDT could induce NF- κ B activation in esophageal cancer cells (as shown in Fig. 6 and Fig. 7).

Studies have shown that NF- κ B activation played a negative role in the process of PDT, such as the anti-apoptotic response, and down-regulation of NF- κ B-sensitized cancer cells to PDT [11-13]. Therefore, it is important to find a proper "sensitizer" to inhibit NF- κ B activation in combination with PDT. We propose DHA, a NF- κ B deactivator, as a potential candidate [14-17]. In our previous study, we demonstrated, for the first time, that DHA reduces the viability of esophageal cancer cells in a dose-dependent manner [17]. Furthermore, inhibition of gemcitabine-induced NF- κ B activation was one of the mechanisms by which DHA dramatically promotes its anti-tumor effect on pancreatic cancer [14], and DHA decreased the luciferase activity of COX-2 regulation-related transcription factors, including NF- κ B, AP-1, C/EBP and CREB, in murine macrophage RAW 264.7 cells [20]. In addition, DHA can inhibit NF- κ B activity in osteosarcoma and Bcr/Abl⁺ chronic myeloid leukemia K562 cells [16, 21]. As shown in Fig. 6 and Fig. 7, and consistent with the studies mentioned above, we found that DHA inactivated NF- κ B and potentiated the anti-tumor effect of PDT on esophageal cancer cells. Our results also indicated that this sensitizing effect originated from the inhibitory effect of DHA upon PDT-induced NF- κ B activation, which has been considered a negative role [11-13]. Furthermore, we are moving forward to investigate the exact molecular mechanism behind this sensitization effect from the aspect of apoptosis.

It is known that Bcl-2, which is a target of NF- κ B, is involved in protection from apoptosis [22]. It has been demonstrated that some agents can activate NF- κ B and stimulate Bcl-2 gene promoter activity in the MIA-PaCa-2 human pancreatic cancer cell line [23]. Bcl-2 is located on the membranes of mitochondria, whereas Bax directly binds to Bcl-2 and inhibits its function. An increase in the ratio of Bax/Bcl-2 stimulates the release of cytochrome c from the mitochondria into the cytosol and cytosolic cytochrome c binding to Apaf-1. These events activate downstream caspase-9 and caspase-3, leading to activation of the mitochondrial apoptosis pathway [24, 25]. Several studies have demonstrated that DHA can induce apoptosis in pancreatic cancer, colorectal cancer, osteosarcoma and others [14-16]. In our previous study, we demonstrated that DHA induces apoptosis by up-regulating the expression of Bax, down-regulating Bcl-2, Bcl-xL and procaspase-3 and increasing caspase-9 activation and induced cell cycle arrest by down-regulating cyclin E, CDK2 and CDK4 [17]. Furthermore, PDT induces apoptosis in esophageal cancer cells [5], hepatic cancer cells [26], pancreatic cancer cells [27] as well as the human head and neck cancer cells AMC-HN-4 [28]. Similarly, in the present study, we found that 5-ALA-PDT induced apoptosis in esophageal cancer cells. As mentioned above, our results showed that PDT or DHA as a single treatment induced apoptosis. However, their combination resulted in a significant increase in the cell apoptosis rate in Eca109 and Ec9706 cells. In our opinion, by abrogating PDT-induced NF- κ B activation, DHA significantly down-regulated Bcl-2, which augmented the induction effect on apoptosis, compared with the single PDT group. Moreover, we found that combined treatment of PDT and DHA down-regulated the expression of Bcl-2 and up-regulated Bax,

thereby reducing the Bcl-2/Bax ratio, and increased caspase-3 and caspase-9 activation, all of which increased apoptosis in both cell types.

In conclusion, our results demonstrate that DHA augments PDT-induced growth inhibition in esophageal cancer cells, and that inactivation of NF- κ B activity is a possible mechanism by which DHA sensitizes esophageal cancer cells to PDT-induced growth inhibition. However, these results are only based on in vitro studies, and further studies in vivo are necessary.

Abbreviations

PDT (photodynamic therapy); NF- κ B (nuclear factor-kappa B); APP (aminopyropheophorbide); 5-ALA (5-Aminolevulinic acid); DHA (Dihydroartemisinin); FBS (fetal bovine serum); PI (propidium iodide); EMSA (Electrophoretic Mobility Shift Assay); PPIX (protoporphyrin IX).

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Disclosure Statement

The authors have no conflict of interest.

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