MiR-31 Downregulation Protects Against Cardiac Ischemia/Reperfusion Injury by Targeting Protein Kinase C Epsilon (PKCε) Directly

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Key Words
Ischemia/Reperfusion injury • miR-31 • PKCε • NFκB

Abstract
Background: Various miRNAs have been shown to participate in cardiac ischemia/reperfusion injury (I/R). miR-31 was identified as the most strikingly upregulated miRNA after acute myocardial infarction; therefore, the underlying role and mechanism of miR-31 in cardiac I/R was investigated. Methods: miR-31 expression was detected after cardiac I/R in mice. The cardioprotective effect of miR-31 downregulation was assessed in vitro and in vivo. The functional target gene and its downstream molecule were determined. Results: miR-31 expression increased after I/R. miR-31 downregulation increased cell viability and SOD activity and decreased LDH activity and MDA content in vitro. The myocardial infarct size in vivo was alleviated by miR-31 downregulation. PKCε was identified as the functional target gene of miR-31, and NFκB was identified as its downstream molecule that was involved in the miR-31-mediated cardioprotective effect. Conclusion: miR-31 expression increased throughout the cardiac I/R process, and miR-31 downregulation induced a cardioprotective effect via a miR-31/PKCε/NFκB-dependent pathway.

Introduction
Cardiac ischemia/reperfusion injury (I/R) is an unavoidable reality after myocardial infarction and represents a major cause of morbidity and mortality in humans [1]. An extraordinary effort has been made to search for novel therapeutic targets to minimize...
I/R injury. Most of the targets identified thus far are proteins. Using a proteomic approach in ischemic rat hearts, target candidate proteins such as ALDH2 have been validated and screened for the development of potential therapeutics [2].

Recently, another potential therapeutic target that has emerged to treat cardiac I/R is microRNA (miRNA). miRNAs are 20-22 nucleotide noncoding RNAs that regulate gene expression at post-transcriptional levels. Accumulating evidence has demonstrated that miRNAs have crucial roles in various cellular and biological processes. Various miRNAs, including miR-1 [3], miR-15 [4], miR-92a [5], miR-320 [6], and miR-494 [7] have been shown to participate in cardioprotection against cardiac I/R. However, the roles of many other miRNAs that are aberrantly expressed after cardiac I/R and the mechanisms involved remain unknown. For instance, although miR-31 was identified as the most strikingly upregulated miRNA after acute myocardial infarction in rats [8], the underlying role and mechanism involved remain uninvestigated.

miR-31 is a broadly conserved miRNA that is expressed in a variety of tissues and cells. Multiple studies have focused on its role in the metastasis of various cancers by downregulating the expression of various oncogenes including RhoA [9, 10] and radixin [10], as well as several integrins, directly [11]. By direct interaction of miR-31 with the 3'-UTR of PKCε mRNA, PKCε has been identified as a functional gene that accounts for human breast cancer cell sensitivity to chemo- and radiotherapy [12]. In this study, we sought to determine the expression mode of miR-31 during the I/R process in mice. Thus, this study found that miR-31 plays a role in cardioprotection by targeting PKCε directly.

Materials and Methods

Cardiac ischemia/reperfusion injury

Standard principles of laboratory animal care were followed. Male C57BL/6 mice (aged 6-8 wk, 18-21 g) were purchased from the Shanghai SLAC Experimental Animal Center (Shanghai, China). After the mice were administered anesthesia (pentobarbital, 80 mg/kg, i.p.), they were placed on a temperature-controlled heating pad. The mice were orally intubated with 20-gauge tube and ventilated. A left-sided thoracotomy was performed, and the heart was exposed. Then, a 7-0 silk thread was passed through the left anterior descending coronary artery (LAD) near its origin. Both ends of the thread were passed through a plastic cannula to allow the ligation and reopening of the coronary artery. Myocardial I/R was conducted by 30 min ischemia and 24 h reperfusion. After reperfusion, a double staining technique using triphenyltetrazolium chloride (TTC) to mark vital and necrotic tissue and Evans blue staining to negatively mark the area at risk (AAR) was used. The location for samples was standardized for all mice. Planimetric determination of the infarct size and the AAR was performed using ImageJ Software version 1.44p. The extent of the infarct sizes was determined by calculating the percentage of infarction compared to that of the AAR from 4-5 discs per heart. Each group of animals consisted of at least 6 mice.

Isolation and culture of neonatal mouse cardiomyocytes and anoxia/reoxygenation

A breeding program was performed to produce neonates. The neonatal cardiomyocytes were prepared according to methods described previously [13, 14]. Briefly, ventricular myocardial tissues from C57BL/6 mice born within 24 h were minced in a nominally Ca²⁺- and Mg²⁺-free Hanks’ balanced solution. Cardiomyocytes were dispersed using 0.625 mg/mL collagenase (type II) at 37°C for 40 min. The isolated cells were preplated for 90 min to remove non-cardiomyocytes. The cardiomyocytes were plated in M199 medium containing 10% fetal calf serum in 35 mm Petri dishes precoated with 1% gelatin. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. After the cardiomyocytes were cultured for 48 h, they were plated in dishes for the following studies.

The cells were incubated at 37°C in an atmosphere with 5% CO₂, 20% O₂, and 75% Ar (standard gas mixture). Anoxia was attained using an airtight jar from which the O₂ was flushed with a gas mixture containing 5% CO₂ and 95% Ar for 30 min. Reoxygenation was realized by exchanging fresh medium and by aerating the medium with the standard gas mixture for 24 h.
RNA extraction, qRT-PCR and northern blot analysis

Total RNA was isolated using a mirVana miRNA Isolation Kit (Ambion). Taqman probes for miR-31 and sno135 were purchased from Applied Biosystems (Foster City, CA, USA). qRT-PCR for miR-31 was performed on cDNA generated from 50 ng of total RNA according to the manufacturer’s protocol. Sno135 was used as an internal control for miR-31 template normalization. The threshold cycle (Ct) was set within the exponential phase of the PCR. The relative gene expression was calculated by comparing cycle times for each target PCR. The target PCR Ct values were normalized by subtracting the sno135 Ct value, which provided the ΔCt value. Then the relative expression level between treatments was calculated using the following equation: relative gene expression = 2\(^{-\text{ΔCt}}\). Northern blot analysis of miR-31 was performed as described previously [15]. The probe sequences used are as follows: miR-31: 5’-CAGCTATGCCAGCATCTTGCC-3’; 5S rRNA: 5’-TTAGCTTCCGAGATCA-3’.

MiR-31 knockdown, miR-31 overexpression, and PKCe downregulation in cultured cardiac myocytes

For miR-31 knockdown, con-anti-miR or anti-miR-31 (Ambion, Austin, TX, USA) was added to the culture media at a final oligonucleotide concentration of 20 nM; for miR-31 upregulation, con-miR or miR-31 mimics (Ambion) were added to the culture media at a final oligonucleotide concentration of 10 nM using RNAiMAX (Invitrogen). PKCe siRNA (5’-UGAAUUUGUGGCUCUUCACCUCAUGTT-3’) and scrambled siRNA (5’-UUCUCCGAACGUGUCACGU-3’), which was used as a negative control, were both synthesized by GenePharma Company (Shanghai, China), and the transfection of siRNA into cells was achieved using Lipofectamine 2000 reagent (Invitrogen).

Oligonucleotide administration in vivo

A chemically modified antisense oligonucleotide (antagomir) specific for miR-31 and a non-specific control oligonucleotide were synthesized by GenePharma Company (Shanghai, China). The sequence of the antagomir against miR-31 is as follows: 5’-mC(s)mAmA(s)mGmCmUmAmUmGmCmAmCmAmUmCmUmUmG(s)mC(s)mC(s)mU(s)-Chol-3’, where m is a 2’-OMe-modified nucleotide, (s) is a phosphorothiate linkage, and Chol is a cholesterol group linked through a hydroxyprolinol linkage. The mice were treated with the oligonucleotides, which were diluted in 0.2 mL saline, at 80 mg/kg body weight through tail vein injection for 3 consecutive days. Saline with a non-specific control oligonucleotide was injected into mice for control purposes.

Cell viability determination

Cell viability was evaluated by the ability to reduce MTT, which is an indicator of metabolic activity. This viability assay was conducted in 96-well plates; MTT reduction was determined by spectrophotometry using a microplate reader (Bio-Tek Instruments, USA).

Measurement of lactate dehydrogenase (LDH), superoxide dismutase (SOD) activity and malondialdehyde (MDA) contents in cells

At 24 hours after anoxia/reoxygenation, the LDH content was detected using a chromotometry assay kit, and the SOD content was measured by spectrophotometry using a commercially available detection kit (both kits were purchased from Nanjing Jiancheng Biochemical Reagent Co., Nanjing, China) according to the manufacturer’s instructions. The MDA content was determined using the thioarbituric acid method with a commercial kit. The absorbance of each supernatant was measured at 532 nm.

Luciferase assay

A fragment of the 3' UTR of PKCe mRNA containing the putative miR-31 binding sequence was cloned into a firefly luciferase reporter construct. The construct with a mutated targeting fragment (GAACGG) at the 3’-UTR of PKCe without the putative miR-31 binding sequence was used as a mutated control. HEK 293 cells were transfected with pre-miR-31 24 h before the transfection of pmirGLO vectors (Promega) possessing either the wild type (PKCe 3’-UTR WT) or the mutant (PKCe 3’-UTR mut) sequence. As controls, cells were either mock-transfected (without miRNA) or transfected with a pre-miRNA negative control.

Adenovirus transduction

To suppress NFκB activation, myocytes were infected with an adenovirus harboring a dominant-negative IκBα mutant (Adv-dnIκBα) or a control empty vector (Adv-Null) (both were purchased from Cell
Biolabs Inc, San Diego, CA, USA). Myocytes were plated, incubated for 4 h, washed with PBS, and subsequently infected with 20 multiplicities of infection of the Adv-dnIkBα or the control Ad-Null in serum-free media. After a 2 h absorption period, the cells were treated with con-anti-miR or anti-miR-31 as indicated. After anoxia/reoxygenation injury, the cells were collected to determine NFκB activation, cell viability, and LDH, SOD and MDA contents.

**Preparation of total protein extracts and purification of nuclear fractions**

The cells were collected, lysed in RIPA lysis buffer (1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, and protease inhibitors in PBS), and then centrifuged at 10,000 revolutions per minute after sonic disruption. The extracts were collected as total protein extracts.

The collected cells were suspended in buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.2 mM EDTA, 1 mM DTT, and PMSF 0.5 mM; 10 mg/mL each of aprotinin, pepstatin, and leupeptin; 50 mM NaF and 1 mM Na$_3$VO$_4$ at 4°C. After 10% Nonidet P-40 was added, the liquid was vortexed for 15 sec and centrifuged at 5000 revolutions per minute. Then, the cell pellets were resuspended in buffer B (20 mmol/L HEPES-KOH pH 7.9, 1.5 mmol/L MgCl2, 400 mmol/L NaCl, 0.2 EDTA, 25% glycerol, 1 mmol/L DTT, 1 mmol/L PMSF, 2 μg/mL aprotinin and 10 μg/mL leupeptin) and gently shaken for 30 min at 4°C. Nuclear protein extracts were obtained by centrifugation at 12,000 revolutions per minute for 15 min. All the extracts were stored at -80°C.

**Electrophoretic mobility shift assay (EMSA)**

The nuclear extracts (20 μg) were pre-incubated for 10 min in binding buffer (1 μg poly dI-dC, 10 mmol/L Tris-HCl (pH 7.5), 50 mmol/L NaCl, 1 mmol/L EDTA, 5% glycerol, 1 mmol/L DTT, and 1 μg/μL BSA) on ice, followed by 30 min of incubation at room temperature with 1×10$^5$ dpm (approximately 0.5 ng) of a γ-$^{32}$P-labeled probe (Amersham Biosciences, Sunnyvale, CA, USA) containing the NFκB binding site 5'-AGTTGAGGGGACTTTCCCAGGC-3' (Santa Cruz Biotechnology, Santa Cruz, CA, USA). DNA-protein complexes were run on a 6% polyacrylamide gel.

**Western blot analysis**

Samples were separated on SDS-polyacrylamide gels and electrotransferred onto nitrocellulose membranes. The membranes were blocked with 3% bovine serum albumin for 2 h and then incubated overnight at 4°C with antibodies against PKCε (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or GAPDH, followed by incubation with HRP-conjugated secondary antibody. The quantitative relative expression (RE) of protein was calculated by normalizing the values to GAPDH protein expression.

**Statistical analysis**

The values are presented as the means ± SD. For relative gene expression, the mean value of the control group was defined as 100%. Samples were analyzed by two-tailed unpaired Student’s t-test. The software SPSS13.0 was used to analyze the experimental data. Statistical significance was defined as P<0.05.

**Results**

**The effect of I/R on miR-31 expression**

RNA was isolated from the mouse myocardium or cultured cardiac myocytes at the indicated time points as shown in Fig. 1. Then, miR-31 expression was determined by qRT-PCR. The expression of miR-31 in mouse myocardium increased at all the measured time points, with a maximum reached after 12 h of reperfusion (Fig. 1A). Cardiac myocytes exposed to anoxia/reoxygenation injury in vitro experience a pathophysiological process similar to myocardial I/R in vivo. qRT-PCR analysis of miR-31 expression in cultured cardiac myocytes confirmed a similar pattern; miR-31 expression was upregulated at all examined time points (Fig. 1B). Northern blot analysis further validated the increase in miR-31 expression with a pattern similar to that of the qRT-PCR analysis and was validated in vivo and in vitro (Fig. 1C, D).
MiR-31 downregulation induced a cardioprotective effect in vitro and in vivo

We demonstrated that cardiac I/R or anoxia/reoxygenation injury was accompanied by increased miR-31 expression. Next, we tested whether miR-31 downregulation induced a cardioprotective effect.

A cell I/R model was applied as described in the Materials and methods section. We found that miR-31 expression was successfully inhibited by anti-miR-31 in cultured cardiomyocytes (Fig. 2A). Then, cardiomyocytes were examined by testing their viability, LDH, SOD activity and MDA content. As shown in Fig. 2B, cell viability increased in the anti-miR-31-pretreated group (P<0.05) compared to the con-anti-miR-pretreated group. Furthermore, cell membrane integrity and necrosis were estimated by the amount of LDH released into the media in response to oxidant burden. ROS-induced toxicity, which is normally accompanied by an increase in lipid peroxides, was determined by measuring MDA content and SOD activity. The anti-miR-31 group presented better cardioprotective effects, which were demonstrated by higher SOD activity, lower LDH activity and MDA content, compared to the con-anti-miR group. The results indicated that miR-31 downregulation has a protective effect against anoxia/reoxygenation injury.

To confirm the miR-31-mediated cardioprotective effect further, an in vivo miR-31 knockdown experiment was performed. The in vivo delivery of anti-miR-31 resulted in an almost 50% reduction in miR-31 expression in mouse myocardium as confirmed by qRT-PCR (Fig. 2C). As expected, the myocardial infarct size was significantly attenuated in anti-miR-31-treated mice compared to that of con-anti-miR-treated mice. Representative TTC- and Evans blue-stained heart slices from these mice are shown in Fig. 2D.

PKCε is a direct target of miR-31 in cardiac myocytes

Computational analysis indicated that PKCε is a potential target gene of miR-31 (Fig. 3A). To verify that PKCε is a target gene of miR-31 in cardiac myocytes, gain and loss of function
approaches were applied. miR-31 overexpression was verified using qRT-PCR as described previously, and we found that miR-31 expression increased in cultured cardiomyocytes upon treatment with miR-31 mimics (Fig. 3B). PKCε expression in cardiac myocytes was upregulated by anti-miR-31 but downregulated by miR-31 mimics (Fig. 3C). The results indicated that PKCε is a potential miR-31 target gene. To confirm that miR-31 binds directly to PKCε and inhibits its expression, we performed luciferase reporter assays. The pmirGLO reporter vector harboring the wild type PKCε 3'-UTR was transfected into HEK293 cells with pre-miR-31, and the luciferase activities of the reporter vector were measured. As expected, pre-miR-31 inhibited the activity of the reporter vector harboring the wild type PKCε 3'-UTR (PKCε 3'-UTR WT) by almost 50% compared with the inhibition of reporter vector activity by the pre-miRNA control. In contrast, reporter vectors containing the mutant PKCε 3'-UTR (PKCε 3'-UTR mut) were not responsive to the miR-31 mimics (Fig. 3D). Taken together, these results suggested miR-31 can downregulate PKCε expression by targeting its 3'-UTR directly.

PKCε is a functional target that accounts for the miR-31-mediated protective effect in cardiac myocytes

To verify whether PKCε is a functional target in the miR-31-mediated cardioprotective effect, we synthesized a short interfering RNA (siRNA) for PKCε to determine its effect on the miR-31-mediated cardioprotective effect. As shown in Fig. 4A, the siRNA against PKCε
sharply decreased PKCε protein expression in anti-miR-31-treated cells. The cardiac myocyte injury, as assessed by cell viability, LDH content, SOD activity and MDA content, revealed a cardioprotective effect caused by miR-31 downregulation (* P<0.05) (Fig. 4B). However, the protective effect induced by anti-miR-31 pretreatment was abolished by PKCε siRNA (# P<0.05) (Fig. 4B).

NFκB is a downstream molecule of PKCε that mediates the observed protective effect in cardiac myocytes caused by miR-31 downregulation

The essential role of increased PKCε and NFκB activation in ischemia preconditioning-induced cardioprotection has been strongly demonstrated [16, 17]. We demonstrated that miR-31-mediated cardioprotection was PKCε-dependent, which suggested a possible role of NFκB activation in miR-31-mediated cardioprotection.

Next, we examined NFκB activation and its role in miR-31-mediated protective effect. As shown in Fig. 5A, we found that NFκB activation increased in the anti-miR-31-treated group compared to that in the con-anti-miR-treated group after anoxia/reoxygenation.
injury. However, this increase was inhibited after pretreatment with siRNA against PKCε (Fig. 5B). The result revealed that NFκB is a downstream molecule of PKCε. To verify the role of NFκB activation in miR-31 signaling, Adv-dnIκBα was adopted for the inhibition of NFκB activation. We found that transfection with Adv-dnIκBα inhibited NFκB activation (Fig. 5C), which, in turn, completely abolished the cardioprotective effect caused by miR-31 downregulation (# \( P < 0.05 \)) (Fig. 5D). In summary, our results demonstrated that NFκB is a downstream molecule of PKCε that mediates the observed protective effect in cardiac myocytes caused by miR-31 downregulation.

**Discussion**

In the present study, we found that miR-31 was upregulated during the cardiac I/R process. Recent studies have revealed that miR-31 plays important roles in tumor metastasis, angiogenesis [18], osteogenesis [19], inflammation [20], and vascular smooth muscle cell proliferation [21]. However, the biological role of miR-31 in cardiac myocytes remains unknown. We demonstrated that miR-31 downregulation induces a cardioprotective effect.

Among the potential targets of miR-31, PKCε has been shown to play an essential role in ischemic late preconditioning [16] and other drug-induced cardioprotection [22]. Furthermore, PKCε has been identified as the functional target gene in human breast cancer cells [12]. Thus, PKCε is the most likely functional target gene of miR-31 in cardioprotection. However, because miRNA-mediated effects on gene expression and cellular functions are cell specific, whether PKCε is a miR-31 target gene in cardiac myocytes was examined. PKCε expression in cultured cardiac myocytes was demonstrated to be regulated by miR-31 via both gain and loss of function approaches. In addition, PKCε was shown to be regulated by miR-31 through direct binding to its 3’-UTR. Finally, PKCε downregulation completely abolished the observed anti-miR-31-mediated cardioprotective effect. Taken together, the
NFκB is a downstream molecule of PKCε that mediates the observed protective effect in cardiac myocytes caused by miR-31 down-regulation. A: Original representative EMSA of NFκB activation in cultured cardiac myocytes that were subjected to I/R injury after being transfected with con-anti-miR or anti-miR-31. B: Original representative EMSA of NFκB activation in cultured cardiac myocytes that were subjected to I/R injury after being transfected with con-anti-miR or anti-miR-31, followed by transfection with scramble siRNA or PKCε siRNA. C: Original representative EMSA of NFκB activation in cultured cardiac myocytes that were subjected to I/R injury after being transfected with adv-Null or adv-dnIκBα, followed by transfection with con-anti-miR or anti-miR-31. D: Cultured cardiac myocytes and media that were subjected to I/R injury after being transfected with adv-Null or adv-dnIκBα, followed by transfection with con-anti-miR or anti-miR-31, were collected for cell viability, LDH, SOD and MDA determination. The data are presented as the means of three separate experiments. * P<0.05 vs con+Null; # P<0.05 vs anti+Null. con + Null, con-anti-miR + Adv-Null; anti + Null, anti-miR-31 + Adv-Null; anti + dnIκBα, anti-miR-31 + Adv- dnIκBα; OP, oligonucleotide/protein complex.

Results indicate that PKCε is a functional target gene of miR-31 that accounts for miR-31-mediated cardioprotection.

NFκB activation is regulated by miR-31 via a PKCε-dependent pathway in human breast cancer cells [12] or via a STK40-dependent pathway in human adult epidermal keratinocytes [20]. The relationship between PKCε and NFκB has also been explored extensively. NFκB activation is modulated by PKCε in adult rabbit cardiomyocytes via mitogen-activated protein kinases [23]. NFκB activation in prostate cancer is mediated by PKCε via facilitating the assembly of the TNF receptor-1 signaling complex [24]. In U87/EGFR cells, NFκB activation is mediated by PKCε via IKKβ kinase activation [25]. In the current study, we demonstrated that NFκB activation is regulated by PKCε in cultured myocardial myocytes, although the underlying mechanism remains undetermined. In addition, we confirmed that NFκB activation is essential for the miR-31-mediated cardioprotection in cultured cardiac myocytes.

In most situations, NFκB is activated by I/R, and the inhibition of NFκB activation results in a cardioprotective effect in cardiac I/R models [26]. However, NFκB activation is an obligatory step in ischemic late preconditioning, and preconditioning-induced cardioprotection is abolished when NFκB activation is inhibited [17]. In response to late
preconditioning, NFκB activates a set of cardioprotective genes and represses several pro-cell death genes. Functional studies have demonstrated that hspa1a, which encodes Hsp70.3, plays a critical role in the NFκB-mediated cardioprotection of late preconditioning against I/R [27]. Anesthetic preconditioning (APC) with sevoflurane produces myocardial protection against I/R. In APC-induced cardioprotection, NFκB activation acts not only as a trigger but also as a mediator that plays an important role by downregulating the inflammatory proteins intercellular adhesion molecule-1 and TNF-α during reperfusion, ultimately decreasing caspase-3 expression and apoptosis [28]. miR-31-mediated cardioprotection was eliminated when NFκB activation was inhibited by Adv-dnIκBα during the I/R process, indicating that different mechanisms may exist in I/R-induced and miR-31/PKCε signaling-induced NFκB activation in cardiac myocytes. However, whether apoptosis and inflammation are involved in the downstream signals of the miR-31/PKCε/NFκB signaling pathway should be determined.

In this study, reperfusion sustained the vast majority of the I/R process, and miR-31 expression increased throughout the I/R process, with peak upregulation at 12 h reperfusion. Therefore, we hypothesized that the observed cardioprotection was achieved primarily during the reperfusion period by miR-31 downregulation, followed by increasing PKCε expression and NFκB activation. Published data have suggested that miRNAs are involved in ischemia preconditioning, miR-21, which was identified as a preconditioning-regulated miRNA after a 6 h ischemic preconditioning stimulus, is also involved in this process, and miR-21 upregulation plays a beneficial role in the I/R process [29]. The modulation of miR-199a expression, which decreased during early ischemic preconditioning in both cultured rat cardiac cells and porcine hearts, also produced cardioprotection [30]. The injection of miRNAs extracted from ischemia preconditioning myocardium also protected the heart against I/R [31]. PKCε and NFκB activation are both necessary steps in ischemia late preconditioning, and their activation is regulated by miR-31-mediated signaling in cardiac myocytes. Therefore, a follow-up study is warranted to determine whether miR-31 is involved in ischemia preconditioning.

In conclusion, we observed that miR-31 expression increased throughout the I/R process and that miR-31 downregulation induced a cardioprotective effect via a miR-31/PKCε/NFκB-dependent pathway. These findings offer promise for the application of miR-31 as a new target for cardioprotection.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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