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Original Paper

NEFA-Sensitive Orai1 Expression in Regulation of *De Novo* Lipogenesis

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Key Words
NEFA • *De novo* lipogenesis • Orai1 • NFκB

Abstract
Background/Aims: Non-esterified fatty acids (NEFAs) are important inducers of inflammatory responses and hepatic lipid accumulation, which lead to non-alcoholic fatty liver disease (NAFLD). High plasma NEFA is found in NAFLD patients, and associated with metabolic syndrome and type-2 diabetes. NFκB is known to upregulate Orai1, the Ca^{2+} channel responsible for store-operated Ca^{2+} entry. The present study explored the role of NEFA-sensitive NFκB-dependent Orai1 expression in the regulation of lipid synthesis. ***Methods:*** BRL-3A rat liver hepatocyte lines were studied in the absence and presence of NEFA. Transcript and protein expression levels of factors involved in lipid synthesis were quantified by quantitative polymerase chain reaction (qPCR) and western blot analyses. Fatty acids were measured by immunofluorescence. ***Results:*** NEFA significantly increased, as indicated by the expression of sterol regulatory element-binding protein 1 (SREBP-1c), fatty acid synthase (FAS), acetyl-CoA carboxylase α (ACC1), Orai1, and NFκB p65 by qPCR and western blot analyses. These effects were reversed by the Orai1 inhibitor, 2-aminoethoxydiphenyl borate, and the NFκB inhibitor, wogonin. Furthermore, SREBP-1c, FAS, ACC1, and Orai1 were significantly decreased by Orai1 silencing. ***Conclusions:*** Taken together, these results demonstrated that NEFA-sensitive NFκB-dependent Orai1 expression regulates *de novo* lipogenesis.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a very common disorder characterized by excessive lipid deposition in hepatocytes (steatosis) with no alcohol consumption [1]. NAFLD contributes significantly to morbidity and mortality associated with obesity, type 2 diabetes, and metabolic syndrome [2, 3]. NAFLD involves increased hepatic fatty acid synthesis, decreased fatty acid catabolism [4], and cellular stress, including oxidative stress [5], inflammatory/immune responses, and endoplasmic reticulum stress [6].

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Normally, non-esterified fatty acids (NEFAs) and triglycerides (TGs) are increased in the plasma of NAFLD patients. The major NEFAs include palmitic, palmitoleic, stearate, oleic, and linoleic acids [7-9]. Previous studies have reported that single fatty acids can influence lipid accumulation. In the present study, a high concentration of NEFAs was prepared to approximate the composition and concentration of circulating NEFAs. Fatty acids in the liver used for TG synthesis are provided by the diet, adipose tissue lipolysis, or *de novo* lipogenesis [10]. Previous reports indicate that sterol regulatory element-binding protein 1 (SREBP-1c), acetyl-CoA carboxylase α (ACC1), and fatty acid synthase (FAS) were important factors in *de novo* lipogenesis [11-13]. However, the mechanisms underlying the regulation of lipid metabolic pathways in the setting of NAFLD have only been partially elucidated. Recent observations showed that lipid accumulation in chronic obesity is stimulated by cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) in the livers of Zucker rats, and that store-operated calcium entry (SOCE) was involved in regulating metabolism [14-16]. A better understanding of the regulation of lipid metabolism in steatotic hepatocytes will provide insight that could lead to the development of new therapeutic strategies for the control of NAFLD.

To the best of our knowledge, the role of the cytosolic $[Ca^{2+}]_i$ in lipid accumulation of NAFLD, especially in *de novo* lipogenesis, remains unknown. In some cell types, Ca^{2+} may enter through SOCE, which is accomplished by the 4-transmembrane-spanning pore forming calcium release-activated channel moiety Orai1 [17-19]. Orai1 has previously been shown to be regulated by nuclear factor NFκB [20, 21].

In patients with NAFLD and type 2 diabetes, excessive amounts of TGs are deposited in hepatocytes leading to steatosis and the activation of the NFκB pathway [22]. The present study explored whether NEFA induces *de novo* lipogenesis on BRL-3A cells and, if so, whether the effect involves NFκB-sensitive Orai1 expression.

Materials and Methods

Cell culture

BRL-3A rat liver cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin under standard culture conditions. Cells were pretreated with 1.2 mM NEFA preparation for 6 h after starvation overnight. Then, 50 μM Orai1 inhibitor, 2-APB (Tocris Bioscience, Bristol, UK), or 100 μM NFκB inhibitor, wogonin (Sigma-Aldrich Corporation, St. Louis, MO, USA), were added for 6 h.

NEFA preparation

The composition and concentration of NEFAs used in this study were chosen to approximate the concentrations of serum NEFAs [23-25]. The stock NEFA (10 mM) solution included oleic acid (4.35 mM), linoleic acid (0.49 mM), palmitic acid (3.19 mM), stearic acid (1.44 mM), and palmitoleic acid (0.53 mM). Stock NEFA solution was diluted in cell culture medium to achieve a final total NEFA concentration of 1.2 mM.

RNA Silencing

For silencing, 3 x 10^5 cells (6-well plate) were seeded 24 h before the experiment and then transfected with 40 pM rat Orai1 siRNA (Shanghai GenePharma Co., Ltd., Shanghai, China) or non-targeting siRNA (Shanghai GenePharma Co., Ltd.) using siRNA-Mate transfection reagent (Shanghai GenePharma Co., Ltd.) according to the manufacturer's protocol.

Quantification of mRNA expression

Total RNA was isolated with Trizol RNA extraction reagent (Invitrogen Corporation, Carlsbad, CA, USA); mRNA was transcribed with Reverse Transcriptase M-MLV (RNase H-) (Takara Bio, Inc., Otsu, Japan) using an oligodT primer. Quantitative RT-PCR was performed on a BioRad iCycler iQTM Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA).

The qPCR reaction mixture contained 2 μl of cDNA, 1 μM of each primer, 10 μl of FastStart Universal

SYBR Green Master (F. Hoffmann-La Roche AG, Basel, Switzerland), and sterile water for a final volume of 20 µl; qPCR conditions were 95°C for 3 min, followed by 40 cycles at 95°C for 10 s and 58°C for 30 s. Calculated mRNA expression levels were normalized to the expression levels of TATA box-binding protein (Tbp) of the same cDNA sample. Gene expression was quantified with the $2^{-\Delta\Delta CT}$ method.

The following primers were used:

Rat Tbp (TATA box-binding protein):
forward (5'-3'): ACTCCTGCCACACCAGCC
reverse (5'-3'): GGTCAAGTTTACAGCCAAGATTCA

Rat Orai1
forward (5'-3'): CGTCCACAACCTCAACTCC
reverse (5'-3'): AACTGTCGGTCCGTCTTAT

Rat Srebp-1c
forward (5'-3'): GACGACGGAGCCATGGATT
reverse (5'-3'): GGGAAGTCACTGTCTTGGTTGTT

Rat Fas1
forward (5'-3'): CTATTGTGGACGGAGGTATC
reverse (5'-3'): TGCTGTAGCCCAGAAGAG

Rat ACC1
forward (5'-3'): TGAGGAGGACCGCATTTATC
reverse (5'-3'): AAGCTTCCTTCGTGACCAGA

Rat NFκB p65
forward (5'-3'): TTCCCTGAAGTGGAGCTAGGA
reverse (5'-3'): CAGTCGAGGAAGACACTGGA

Western blot analysis

Orai1, NFκB p65, SREBP-1c, FAS and ACC1 relative protein abundances were determined in BRL-3A rat liver cells. The cells were washed in ice-cold PBS. RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) was added to solubilize the cells. Samples were incubated on ice for 30 min and then centrifuged at 14, 000 rpm and 4°C for 20 min. The supernatant was removed and used for Western blotting. Total protein (40-60 µg) was separated by SDS-PAGE, thereafter transferred to PVDF membranes and blocked in 5% non-fat milk/Tris-buffered saline/Tween-20 (TBST) at room temperature for 1 hour. Membranes were probed overnight at 4ºC with polyclonal rabbit or mouse antibodies against Orai1, SREBP-1c, ACC1, FAS, or NFκB p65 (1:700 to 1:1000 in TBS containing 5% BSA and Tween 20). After incubation with HRP-labeled goat anti-rabbit or mouse secondary antibody (Beyotime Biotechnology, 1:2000) for 1 hour at room temperature, bands were visualized with enhanced chemiluminescence reagents (Beyotime). Membranes were also probed with β-Actin antibody (Cell signaling, 1:2000) as loading control. Densitometric analyses were performed using Image J software.

TG content *assay*

After treatments, the cells were collected. The TG content in BRL-3A cells was determined using an enzymatic method with the TG assay kit (Applygen Technologies Inc., Beijing, China) in accordance with the manufacturer's instructions.

Lipid droplet fluorescence assay

BRL-3A cells were treated with 1.2 mM NEFA for 6, 9, or 12 h for assessment of lipid droplet fluorescence by cellular staining with BODIPY 493/503 (Invitrogen Corporation) according to the manufacturer's instructions. Coverslips were mounted with glycerinum and images were obtained with a confocal laser-scanning microscope (Leica TCS SP8; Leica, Wetzlar, Germany) with 40×/1.3 NA differential interference contrast and analyzed with the instrument's software.

Statistical analysis

Data are presented as the means ± standard error of the mean (SEM) with *n* representing the number of independent experiments. All data were tested for significance with the unpaired Student's *t*-test or one-way analysis of variance (ANOVA) followed by the Tukey post-hoc test. A probability (p) value of < 0.05 was considered statistically significant.

Results

The present study explored hepatocyte *de novo* lipogenesis in the presence of high concentrations of NEFA and the involvement of NFκB and Orai1 in this process.

We first investigated the effect of high-concentration NEFA on lipid accumulation and secretion in BRL-3A cells. As illustrated in Figs. 1A and 1B, incubation with 1.2 mM NEFA for 12 h caused extensive lipid droplet formation and increased TG synthesis (Fig. 1C).

The next series of experiments explored whether SOCE via Orai1 and NFκB p65 are induced in BRL-3A cells in response to NEFA, and therefore may participate in the regulation of

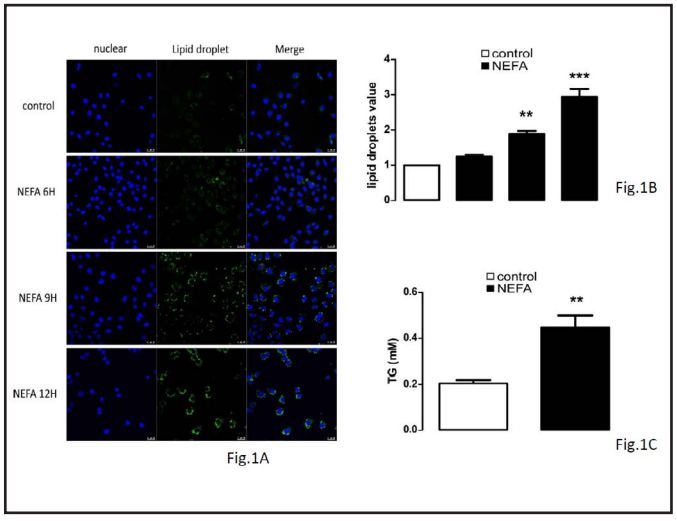

Fig. 1. Effect of high concentration NEFA on lipid accumulation in BRL-3A cells. A. Representative immunofluorescence images demonstrating nuclear staining (DAPI, Blue; left images), lipid droplets staining (Green; middle images), and an overlay of both (merge, right images) in BRL-3A cells incubated without or with different time NEFA (1.2 mM). B. Arithmetic means ± SEM (n=3) of lipid droplets value (relative to control) in BRL-3A cells incubated without or with different time NEFA (1.2 mM). C. Effects of NEFA (1.2 mM, 12h) on TG synthesis in BRL-3A cells. **(p<0.01), ***(p<0.001) indicate difference from control (t-test).

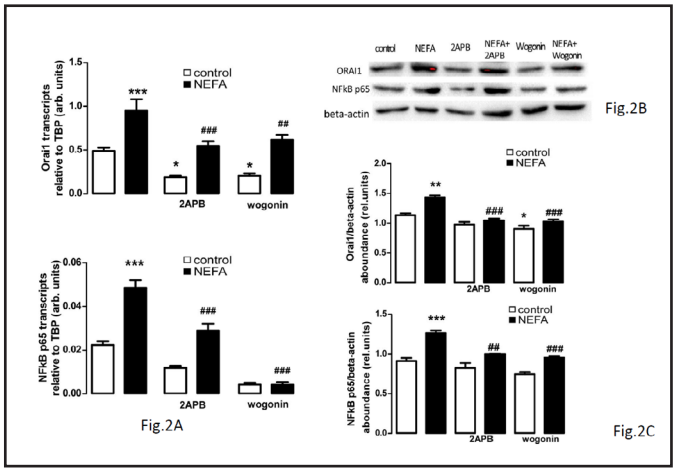

Fig. 2. Effect of high concentration NEFA on Orai1, p65 in BRL-3A cells. A. Arithmetic means ± SEM (n=12) of Orai1 and NFκB p65 mRNA abundance (relative to Tbp mRNA) in BRL-3A cells incubated without (white bar) or with (black bar) NEFA (1.2 mM, 12h). B. Representative western blots showing the protein expression of Orai1, NFκB p65 and β-actin in BRL-3A cells incubated without or with NEFA (1.2 mM, 12h). C. Arithmetic means ± SEM (n=6) of the Orai1/β-actin and NFκB p65/β-actin ratios in BRL-3A cells incubated without (white bar) or with (black bar) NEFA (1.2 mM, 12h). *(p<0.05), **(p<0.01), ***(p<0.001) indicate difference from control. # (p<0.05), ## (p<0.01), ### (p<0.001) indicate difference from NEFA alone (one-way ANOVA).

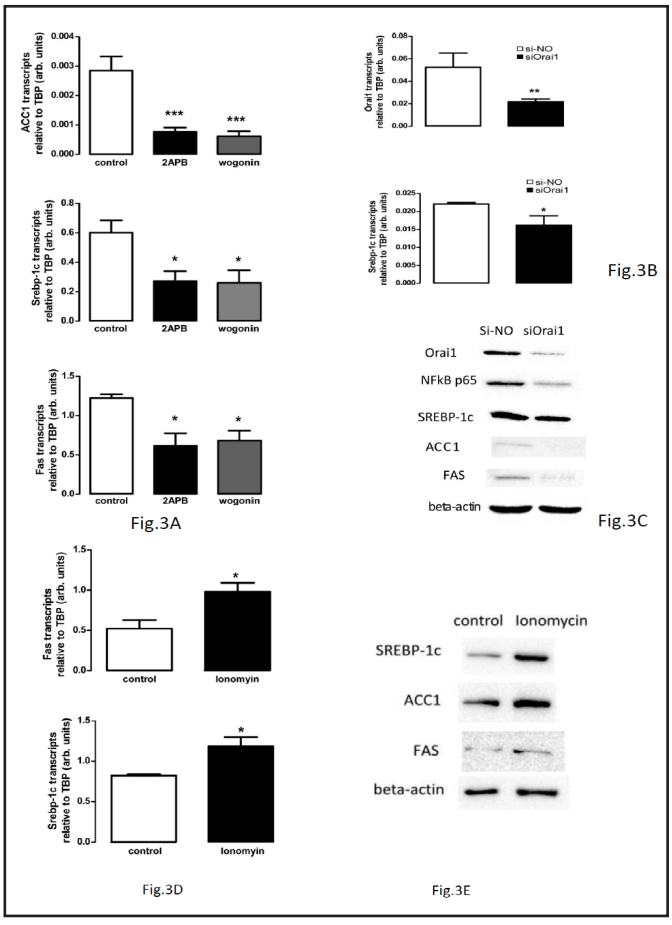

Fig. 3. Effect of Ionomycin and Orai1 silencing on de novo lipogenesis in BRL-3A cells. A. Arithmetic means ± SEM of Srebp-1c, Fas and ACC1 mRNA transcription (relative to Tbp mRNA) in BRL-3A cells incubated without (white bars) or with (black bars) Orai1 inhibitor 2APB (100 nM, 6h, n=12), (grey bars) NFκB inhibitor Wogonin (500 nM, 6h, n=12), respectively. B. Arithmetic means ± SEM of Srebp-1c and Orai1 mRNA transcription (relative to Tbp mRNA) in BRL-3A cells incubated without (white bars) or with (black bars) siOrai1. C. Representative western blots showing the protein expression of SREBP-1c, FAS and ACC1, Orai1, NFκB p65 and β-actin in BRL-3A cells incubated without (Si-NO) or with siOrai1. D. Arithmetic means ± SEM of Srebp-1c, and Fas mRNA transcription (relative to Tbp mRNA) in BRL-3A cells incubated without (white bars) or with (black bars) ionomycin (100 nM, 2 h, n=15). E. Representative western blots showing the protein expression of SREBP-1c, FAS and ACC1 and β-actin in BRL-3A cells incubated without or with ionomycin (100 nM, 2 h, n=9). *($p<0.05$), **($p<0.01$), ***($p<0.001$) indicate significant difference (t-test).

de novo lipogenesis. As illustrated in Fig. 2, expressions of mRNA and protein of Orai1 and NFκB p65 were dramatically up-regulated by NEFA treatment and these effects were reduced by the Orai1 inhibitor, 2APB, and the NFκB inhibitor, wogonin. Moreover, transcription levels of SREBP-1c, ACC1, and FAS were significantly reduced by both 2APB and wogonin, respectively (Fig. 3A). As illustrated in Figs. 3B and 3C, mRNA and protein levels of *de novo* lipogenesis were significantly decreased by siOai1. To verify the silencing efficiency, Orai1 transcript and protein levels were quantified, which showed that both were dramatically decreased in cells transfected with siOrai1. Furthermore, mRNA and protein levels of *de novo* lipogenesis were significantly increased by the Ca^{2+} ionophore ionomycin (Figs. 3D and 3E)

Finally, we determined whether SOCE and/or NFκB are required for lipid accumulation in the presence of high NEFA concentrations. Since Orai1 entry into BRL-3A cells could be attenuated by the NFκB inhibitor wogonin, we tested whether *de novo* lipogenesis was also NFκB-sensitive. Indeed, the transcript and protein levels of SREBP-1c, ACC1, and FAS were reduced by the NFκB inhibitor wogonin and the Orai1 inhibitor 2APB (Fig. 4).

Discussion

The present observations disclose a decisive role of NFκB-Orai1 on *de novo* lipogenesis. The results showed that high concentrations of NEFA promoted *de novo* lipogenesis, which resulted in abnormal hepatic lipid accumulation. According to the present observations, the transcript and protein expression levels of SREBP-1c, ACC1, and FAS were dramatically increased by NEFA

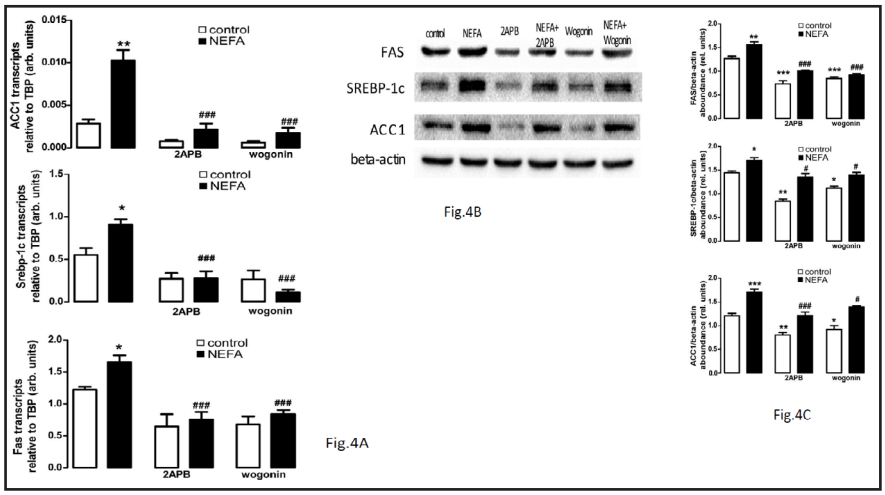

Fig. 4. Effect of NEFA on de novo lipogenesis in BRL-3A cells with or without presence of 2-APB and Wogonin. A. Arithmetic means ± SEM (n=12) of Srebp-1c, Fas and ACC1 mRNA abundance (relative to Tbp mRNA) in BRL-3A cells incubated without (white bars) or with (black bars) NEFA (1.2 mM, 12h) in the absence (left bars) or presence (right bars) of Orai inhibitor 2-APB (100 nM) or of NFκB inhibitor Wogonin (500 nM). B. Representative western blots showing the protein expression of SREBP-1c, FAS and ACC1 and β-actin in BRL-3A cells incubated without (white bar) or with (black bar) NEFA (1.2 mM, 12h) in the absence (left bars) or presence (right bars) of Orai inhibitor 2-APB (100 nM) or of NFκB inhibitor Wogonin (500 nM). C. Arithmetic means ± SEM (n=6) of the SREBP-1c/β-actin, FAS/β-actin and ACC1/β-actin ratios in BRL-3A cells incubated without (white bar) or with (black bar) NEFA (1.2 mM, 12h) in the absence (left bars) or presence (right bars) of Orai inhibitor 2-APB (100 nM) or of NFκB inhibitor Wogonin (500 nM). *, **, *** (p<0.05, p<0.01, p<0.001) indicates difference from control. #, ##, ### (p<0.05, p<0.01, p<0.001) indicates difference from NEFA alone (one-way ANOVA).

treatment during starvation. These effects were virtually abrogated by the inhibition of NFκB and Orai1. The present observations do not rule out involvement of further signals triggered by the NFκB-Orai1 pathway. The regulation of *de novo* lipogenesis by NFκB-Orai1 is expected to influence cellular ionic metabolism.

The present study also showed that transcript and protein expression levels of SREBP-1c, ACC1, and FAS were significantly increased by ionomycin, but decreased by siOrai1, indicating that Orai1 may contribute to *de novo* lipogenesis by promoting the transfer of NFκB p65, which results in the release of pro-inflammatory factors and subsequent induction of liver injury. Indeed, saturated fatty acids can activate the NFκB pathway. Moreover, oleic acid or starvation can induce SOCE and maintain a sustained elevation in intracellular Ca^{2+} levels [16, 26].

SREBP-1c, a membrane-bound transcription factor, promotes TG secretion and lipid droplet formation by stimulating expression of genes involved in fatty acids synthesis. FAS is an important enzyme involved in the *de novo* synthesis of long-chain fatty acids by converting the acetyl-CoA into palmitate, which is subsequently esterified into TGs in the liver. When energy is available, hepatic fatty acids are esterified into glycerol and stored as triacylglycerol or secreted as very low-density lipoproteins. ACC, the rate-limiting enzyme that catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA, is the pivotal enzyme in the biosynthesis of long-chain fatty acids [27]. Previous studies have shown that dietary fat causes synthesis of hepatic fatty acid synthesis, including SREBP-1c, ACC1, and FAS [28]. Moreover, the impairment of lipid metabolism via the inflammatory signaling of toll-like receptor 4 and NFκB has been reported in high fat diet-induced NAFLD [29]. High concentration NEFA causes several inflammatory-based diseases, such as cardiovascular disease, diabetes, and NAFLD by regulating *de novo* lipogenesis [30, 31]. In view of the present observations, it is tempting to speculate that the remarkable increase in *de novo* lipogenesis by high dietary fat or starvation results from NFκB-dependent SOCE via Orai1.

Plasma NEFA, as the major source of fatty acids for TG synthesis, causes abnormal lipid accumulation, which leads to the development of NAFLD [32, 33]. In the present study, the content of TG was measured with an enzyme-linked immunosorbent assay, which indicated that NEFA stimulated TG synthesis and secretion or insulin resistance, resulting in the accumulation of TG. Hence, the NFκB-Orai1 pathway may contribute to TG accumulation.

In conclusion, the present study demonstrated for the first time the involvement of NEFA-sensitive NFκB-Orai1 signaling in the regulation of *de novo* lipogenesis in BRL-3A cells, which included SREBP-1c, FAS, and ACC1.

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Disclosure Statement

The authors of this manuscript have no conflict of interests to declare.

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