Basic Science

Secondhand Smoke Exposure Enhances Cardiac Fibrosis Effects on the Aging Rat Hearts

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Background: Examining aging rats exposed to secondhand smoke (SHS) engenders changes in left ventricular remodeling due to age- or disease-dependent alterations.

Methods: Rats were placed in whole-body exposure chambers and exposed to 10 cigarettes. Filtered air was introduced into the chamber at a low rate. Rats were exposed to SHS for 30 min, twice a day, 5 days per week for 1 month. After 4 weeks SHS exposure, rats were sacrificed for morphological study with trichome staining and left ventricular remodeling related protein analysis using western blot.

Results: Characteristic fibrotic morphology in the left ventricle increased significantly with aging and exposure to SHS. Exposure to SHS elevated TGFβ1/p-Smad2/3/CTGF and MMP2/MMP9 protein expression levels (p < 0.05). No significant differences in FGF-2 and UPA protein expression were noted as a result of SHS exposure. However, TIMP-1, TIMP-2, TIMP-3 and TIMP-4 protein expression were suppressed by SHS exposure. We also observed increased TGFβ1/p-Smad2/3/CTGF (p < 0.01), FGF-2/UPA (p < 0.05) and decreased TIMPs protein expression levels. Corresponding MMP2 and MMP9 upregulation occurred with aging and exposure to SHS. TGFβ1/p-Smad2/3/CTGF and FGF-2/UPA protein expression from SHS exposure were higher than that from aging. In contrast, MMP2 and MMP9 were increased in aging rats compared with SHS exposed rats (p < 0.05); however, TIMP-1 (p < 0.01), TIMP-2 (p < 0.01) and TIMP-3 (p < 0.05) were decreased. TIMP-4 protein expression levels were decreased compared with SHS exposed rats (p < 0.01).

Conclusions: Aging and SHS exposure in rats will produce elevated fibrosis. Exposure to SHS will accelerate aging and left ventricular fibrosis.

Key Words: Aging • Basic fibroblast growth factors • Connective tissue growth factor • Extracellular matrix • Secondhand smoke exposure

INTRODUCTION

Secondhand smoke (SHS) exposure causes various cardiovascular disease risk factors.1 It is well-known that cardiovascular diseases are the major causes of morbidity and mortality in smokers and those exposed to cigarette smoke environments.2 Since 1995 many second-hand smoke cardiovascular system researches studies have been published. Recent studies have found rapidly accumulating evidence that SHS and aging impairs endothelial function3 and cardiovascular system-platelet thrombus formation. Endothelial dysfunction induces direct damage to the endothelium, producing arterial
stiffness, increasing oxidative stress, inflammation, vulnerability to infection, and infarct size. The mechanism explaining the association between aging and SHS exposure with coronary artery disease risk have not been well examined. Statistical study data shows that 25.7% of the elderly are exposed to SHS at home, 34.2% outside of the home and 18.3% exposed to SHS both at home and outside of the home. The vast bulk of SHS exposure is harmful, causing human disease, especially in the elderly.4 Old age is a strong predictor for heart disease morbidity.5 SHS exposure is a complex chemical mixture from the lit end of burning cigarettes. Side stream cigarette smoke toxicity increases with aging and exposure duration. SHS exposure is a known cause of cardiac remodeling with a decrease in left ventricular functional capacity. This study compares the SHS pollutant exposure effect on young and old rats, and determines whether age-related differences exist in modulated left ventricular hypertrophy (LVH) and cardiac remodeling. Cardiac aging is a human physiological change characterized by slowly progressive biological structural changes and functional decline with age in the absence of major cardiovascular risks. The physiological changes in cardiac aging include LVH, increased cardiac fibrosis and valvular degeneration. However, cardiovascular disease is a major risk factor for cause of death in the aged. SHS exposure also leads to cardiovascular diseases such as heart failure and atherosclerosis. Continued SHS exposure always leads to human pathological cardiac hypertrophy. The complications in this environment for the aged are still unclear. SHS exposure may first induce a cardiac hypertrophy phase, especially in the left ventricle. LVH has been observed in rabbits exposed to SHS. LVH leads to ventricular remodeling and increases the risk for a cardiovascular event and mortality.6 However, old age is a significant risk factor for cardiovascular diseases (CVDs).7 Remodeling of the aging left ventricle typically involves a large net loss in active cardiac myocytes and increased accumulation of connective tissue.8 The heart remodeling that occurs with advancing age may be in response to LVH and the increased wall stress and fibrosis observed with early heart failure.9 The normal aged heart presents changes that mimic cardiac disease. Age-related diseases are also accompanied by fibrotic area augmentation and muscle fiber architectural rearrangements in the ventricular myocardium.10 The extracellular space in the left ventricular is now an essential dynamic participant in remodeling.11 Extracellular matrix (ECM) remodeling is an essential process leading to cardiac fibrosis.12 Cardiac fibrosis is the consequence of equilibrium disruption between the synthesis and degradation of collagen molecules.13 Myocardial fibrosis results in an excessive accumulation of collagen fibres.14 At the same time, the mechanism responsible for left ventricular fibrosis in the senescent is unclear. However, these myocardial mechanical properties result from collagen alteration. Myocardial collagen is affected by the aging process.15 The matrix metalloproteinases (MMP 2 and MMP 9) are responsible for extracellular collagen degradation and remodeling. The roles of transforming growth factor β-1 (TGF-β-1) and MMPs (MMP 2 and MMP 9) in left ventricular remodeling are intertwined. Both proteins are complex for fibrosis and both concentrations are elevated. However, MMP activities are regulated by tissue inhibitors of metalloproteinases (TIMPs).16 TGF-β1 is inducted through the connective tissue growth factor to up-regulate pro-fibrotic proteins.37

The present study was conducted to understand and determine the age-related differences between young and old rats after exposure to SHS.

METHODS

Animals

We purchased male rats both young and old in age (young: 6 weeks of age; body weight, 132.5 ± 4.61 g; old: 18 months of age, body weight, 130.2 ± 6.04 g) from the National Science Council Animal Center. The animals were housed in individual cages in an environment controlled animal room, in temperature and humidity controlled chambers. Water and chow were provided ad libitum. All animals were handled according to the Taiwan Society for Laboratory Animals Sciences guidelines for the care and use of laboratory animals.

Experimental groups design and SHS exposure

Rats were divided into 2 age groups, young adult and old male, which were divided into two subgroups. Rats were treated for 4 weeks with SHS exposure as follows: 1) control (C), comprising 6 animals not exposed to cigarette secondhand smoke; and 2) Secondhand
smoking rats (S), comprising 6 animals exposed to cigarette SHS. The rats were placed in whole-body exposure chambers and exposed to 10 cigarettes. Filtered air was introduced into the chamber at a low rate. Rats were exposed to SHS smoke for 30 min, twice a day, 5 days/week for 1 month. Room temperature was maintained at 22-25 °C, and relative humidity was approximately 40%.

**Tissue extraction**

After 4 weeks SHS exposure, all rats were anesthetized with diethyl ether and sacrificed by cervical dislocation. After removal from the thorax the hearts were cleaned with double-distilled water and dried before weighing. The left and right atria and the right ventricular were then removed, and the left ventricular was kept. Left ventricular tissue extracts were obtained by homogenizing the left ventricular samples in phosphate-buffered saline (PBS) at a concentration of 0.1 g tissue/1 mL PBS for 5 min. The homogenates were placed on ice for 10 min and then centrifuged at 12,000 rpm for 30 min. The supernatant was collected and stored at -80 °C for further experiments.

**Masson’s trichrome staining**

Left ventricle cross sections were cut 10 um thick and placed on slides. The slides were then prepared using deparaffinization and dehydration. Slides were passed through a series of graded alcohols from 100% to 95% to 75%, 15 min each. Sections were stained with Masson's trichrome, and then incubated for 1 min at room temperature. After rinsing with water, each slide was then soaked with 85% alcohol, 100% alcohol for 15 min. Stained sections were then rinsed with PBS and air dried before mounting.

**Protein contents**

We determined the protein content of the left ventricle cardiac tissue extract utilizing the Bradford protein assay using the protein-dye kit. We used a commercially available bovine serum albumin as a standard. Changes in optical density were monitored at 595 nm.

**Western blot**

We prepared the tissue extract samples (40 μg) as described above. SDS-PAGE was carried out with polyacrylamide gels. The samples were electrophoresed at 100 V for 1 hr. Electrophoresed proteins were transferred to polyvinylidene difluoride (PVDF) membranes at 150 mA for 2 hr. We incubated PVDF membranes in blocking buffer (5% non-fat milk in PBS-Tween) for 1 hr at room temperature. Polyclonal antibodies against TGF-β1, p-Smad2/3, connective tissue growth factor (CTGF), basic fibroblast growth factors (FGF-2), urokinase-type plasminogen activator (UPA), MMP 2, MMP 9, TIMP-1, TIMP-2, TIMP-3 and TIMP-4 (Santa Cruz) were diluted 1:200 in antibody buffer (TBS). Incubations were performed at room temperature for 12 hr. We washed the immunoblots three times in 5 ml PBS-Tween for 10 min and then immersed in the second antibody solution for 1 hr and diluted 1,000-fold in binding buffer. The filters were then washed in blotting buffer for 10 min three times. Color development was presented in electrochemiluminescence (ECL).

**Quantification of western blot**

The intensity (area x density) of the individual bands on western blot were measured using densitometry. The background was subtracted from the calculated area.

**Echocardiography**

After 4 weeks of exposure treatment, all animals underwent echocardiographic study according to the previously described method. Animals were anesthetized with ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (1 mg/kg). Transthoracic echocardiography was performed at 4 weeks after SHS exposure using a Hewlett-Packard Sonos 5500 ultrasound machine with a 7.5-15 MHz linear-array transducer, as previously described. In the short-axis parasternal view we could obtain a transverse left ventricular one-dimensional image, the ultrasound beam right below the mitral valve plane between the papillary muscles using the 2-dimensional image as a guide for positioning. M-mode image was recorded and analyzed offline. The following parameters were measured and calculated: left ventricular interior diastolic diameter (LVIDd), left ventricular interior systolic diameter (LVIDs), left ventricular interventricular septum at systolic and diastolic (LVIDs, LVIDd), left ventricular posterior wall thickness at systolic and diastolic (LVPWs, LVPWd), fractional shortening (FS%) and ejection fractional (EF%).
Statistical analysis

All data examined were expressed as mean ± standard error of the mean (SEM). Quantitation was carried out for western blot analysis by scanning and analyzing the intensity of the hybridization signals using the FUJIFILM Imagine program. Statistical data analysis was performed using the SigmaStat software. Comparison between groups was made using the two-way analysis of variance (ANOVA) test. The existence of mean differences for age and SHS exposure effects was determined. A p value less than 0.05 and 0.01 was considered statistically significant.

RESULTS

Cardiac structural remodeling alterations in SHS exposure subjects is age-dependent

SHS exposure has been found to be associated with various CVD risk factors. To investigate whether SHS exposure resulted in cardiac remodeling and dysfunction we observed changes in cardiac histological and morphological function using hematoxylin-eosin (Figure 1) and Masson’s trichrome staining (Figure 2). Substantial collagen connective tissue enlargement existed as a result of aging and in aged SHS exposed rats (yellow arrows). The left ventricular interstitial muscle fibers were found broad and disordered (Figure 1A). M-mode parasternal short-axis echocardiographic views of systolic and diastolic system were performed (Figure 1B). Left ventricular Masson’s cross section results showed collagen accumulation and ECM deposition in male young SHS exposure (MYS), male old control (MOC), and male old secondhand smoke (MOS) rat hearts (yellow arrows) (Figure 2A). Young control (MYC), and aging rat hearts exhibited morphological and pathophysiology cardiovascular changes predisposed to LVH or

Figure 1. Characteristic morphological changes in the left ventricles. (A) Changes of histological and morphological cardiac aging exposure to SHS using hematoxylin-eosin stained. Increased extracellular space and disorder cardiac fiber arrangement were observed in MYS, MOC and MOS rat hearts. Scale bar represents 20 μm. (B) Representative M-mode echocardiography taken short-proximal to the left ventricle from MYC, MYS, MOC and MOS rat hearts. Solid line (d), left ventricular free wall endocardial surface at the diastolic system. Solid line (s), left ventricular free wall endocardial surface at the systolic system. All results described are from male young control (MYC), male young secondhand smoke (MYS), male old control (MOC), and male old secondhand smoke (MOS) groups as outlined in experimental design.
heart failure. Left ventricular cross sections from aged SHS exposure exhibited more age-related remodeling. We found the left ventricle cardiac remodeling more prevalent than other parts of the whole heart. In order to determine whether cardiac remodeling occurred in the hypertrophied aging rat hearts left ventricular cross sections were stained with Mason’s trichrome for fibrosis location morphology and identification. We focused on the left ventricular as age-related cardiac remodeling is more prevalent in the left ventricular than other parts of the heart. Collagen accumulation was observed in the Masson’s trichrome stained blue areas (Figure 2A). However, the yellow arrows showed more in the male old SHS exposure (MOS). Measuring the quantity of tissue section area (%), all data was increased in aging (MOC) and aged SHS exposure (MOS) (p < 0.05 and p < 0.01, respectively) (Figure 2B). We observed dramatic remodeling in the left ventricular in aged groups including SHS exposure, when compared with left ventricular sections from the young animals. Substantial collagen connective tissue enlargement existed resulting from aging and SHS exposure in rats (Figure 2). Remarkably, the left ventricular cross sections from the aging rats exposed to SHS group exhibited more age-related remodeling.

Changes in TGF-β1/p-Smad2/3/CTGF activity explain the aging and disease dependent fibrosis alterations

In order to test the aging and SHS exposure effects on connective fibrosis tissue, we examined TGF-β1, p-Smad2/3 and CTGF expression in male young control (MYC), male young SHS exposure (MYS), male old control (MOC), and male old secondhand smoke (MOS) rat hearts (Figure 3). The CTGF expression is regulated directly by TGF-β1 activated p-Smad2/3. Therefore, TGF-β1 protein expression was similar to CTGF and often overexpressed in fibrotic disease. We found that TGF-β1 and p-Smad2/3 expression were increased in aging (MOC) and aging exposed to SHS (MOS), when compared with the young group (MYC) (Figure 3). In aging hearts, CTGF protein expression was substantially incremental compared with MYC. When compared with the young adults the MOS group presented the highest TGF-β1, p-smad2/3 and CTGF protein expression using western blotting analysis (p < 0.01). Indeed, TGFβ1, p-smad2/3 and CTGF protein expression were substantially incremental in the MOC group (p < 0.05). Given the above evidence that TGFβ1, p-smad2/3 and CTGF were up-regulated in tissue fibrosis and collagen accumulation, it should be noted that those increases may be aging effects. Similarly, in aging hearts exposed to SHS, TGFβ1, p-smad2/3 and

Figure 2. Pathological changes in the left ventricles. (A) Fibrosis occurred by collagen location accumulation (blue color) was evident in the left ventricular using Masson’s trichrome staining in MYS, MOC and MOS rats. Scale bar represents 500 μm. (B) Quantitative analysis of collagen content (% tissue section area). Collagen content (%) determined from histological sections expressed as mean ± S.E.M. * significant difference (p < 0.05), ** significant difference (p < 0.01) from the male young control (MYC). All results described are from male young control (MYC), male young secondhand smoke (MYS), male old control (MOC), and male old secondhand smoke (MOS) groups as outlined in experimental design.
CTGF were concomitantly increased compared with the not exposed aging group. Those were aging effects. On the other hand TGF-β1, p-smad2/3 and CTGF in aging hearts exposed to SHS exhibited exacerbated protein expression (p < 0.01).

Changes to FGF2/UPA activity explain the aging-dependent alterations in left ventricular remodeling

Urokinase-type plasminogen activator production through FGF-2 -induces to the stimulation of fibroblast proliferation. To investigate fibroblasts modulating the balance between synthesis and degradation regulated extracellular matrix protein turnover, we examined FGF-2 and UPA protein expression in MYC, MYS, MOC and MOS rat hearts (Figure 4). We examined FGF-2 and UPA protein expression in young, aging and SHS exposed young and old hearts. We found that FGF-2 and UPA protein levels were significantly higher in aging (MOC), and aging exposed to SHS groups (MOS) (p < 0.05). It is worth noting that FGF-2 and UPA protein expression

Figure 3. Cytokines, TGF-β1 and CTGF, and the activation of its receptors, the Smad2/3 phosphorylation protein expression levels are elevated in aging and aging SHS exposure rats. (A) TGF-β1, p-Smad2/3 and CTGF protein expression levels were examined using western blot analysis in left ventricular samples from MYC, MYS, MOC, and MOS rats. (B) Data were quantified in densitometry and expressed as means ± S.E.M. Matched controls to α-tubulin blots are displayed with the protein blots. * significant difference (p < 0.05), ** significant difference (p < 0.01) from the male young control (MYC). All data described are from male young control (MYC), male young secondhand smoke (MYS), male old control (MOC), and male old secondhand smoke (MOS) groups of rats as outlined in experimental design.

Figure 4. UPA and FGF-2 protein expression are elevated in aging and aging SHS exposure rats. (A) Protein expression levels of UPA and FGF-2 using western blot in left ventricular samples from MYC, MYS, MOC, and MOS rats. (B) Data were quantified in western blot results densitometry and expressed as means ± S.E.M. Matched controls to α-tubulin blots are displayed with the protein blots. * significant difference (p < 0.05) from the male young control (MYC). All data described are from male young control (MYC), male young secondhand smoke (MYS), male old control (MOC), and male old secondhand smoke (MOS) groups of rats as outlined in experimental design.
levels in the SHS exposed groups compared with the young control were not significantly different. Aging rat hearts exposed to SHS presented significantly increased protein expression levels (p < 0.05). Urokinase-type plasminogen activator production is through FGF-2 inducers to stimulate fibroblast proliferation. One possibility is that exposure to a range of low smoke concentrations, significantly increased FGF-2 and UPA protein levels only in aging hearts. No significant exposure to SHS effect was observed in the young group.

**SHS exposure accelerated age-dependent MMP 2 and MMP 9 activity increases**

Fibrosis occurs from changes in the balance between extracellular matrix component synthesis and degradation. MMPs are an endogenous enzyme system responsible for degrading all collagen components and turning over the extracellular matrix. Fibrosis occurs from changes in the balance between extracellular matrix component synthesis and degradation. Whether ECM turnover regulation in aging and SHS exposed rat hearts induced cardiovascular disease was investigated. MMP 2 and MMP 9 activity in left ventricular tissues were assessed using western blotting. We sought to determine whether age-related collagen accumulation inducing fibrosis could be related to changes in MMP 2 and MMP 9 regulation. Up-regulation of MMP 9 and MMP 2 in SHS exposed hearts was obtained on aging protein levels using western blot. These results suggest that MMP 2/MMP 9 (gelatinase) contribute to extracellular matrix remodeling in left ventricle fibrosis. No significant differences were found in young rats. During aging, SHS exposure led to increased MMP 2 and MMP 9 activity. Moreover, MMP 2 and MMP 9 protein levels in the left ventricle were significantly higher with aging heart exposure to SHS (MOS) (p < 0.05) (Figure 5). The aging effects in the aging heart exposure to SHS group (p < 0.05).

**Reduced TIMPs expression is present in secondhand smoke exposure and aging rats**

MMP activity is controlled by their inhibitors, TIMPs. The next series of experiments sought to determine whether TIMP levels were altered by aging or SHS exposure. We assessed TIMP-1, -2, -3 and -4 expression using Western blot analysis (Figure 6) to determine whether SHS exposure might modulate cardiac matrix remodeling. We measured potential changes in TIMP-1, TIMP-2,

![Figure 5](image_url)

**Figure 5.** MMP 2 and MMP 9 protein expression only elevated in SHS exposure to old rats. (A) MMP 2 and MMP 9 protein expression were examined by western blot in left ventricular samples from MYC, MYS, MOC, and MOS rats. (B) Data were quantified in densitometry by western blot. Results are mean ± S.E.M. Matched controls to α-tubulin blots are displayed with the protein blots. * significant difference (p < 0.05) from the male young control (MYC). All data described are from male young control (MYC), male young secondhand smoke (MYS), male old control (MOC), and male old secondhand smoke (MOS) groups of rats as outlined in experimental design.
TIMP-3 and TIMP-4 as a function of aging and exposure to SHS in the left ventricles. We found that TIMP-1, TIMP-2, TIMP-3 and TIMP-4 protein levels were reduced in either the SHS exposure or aging groups. These results were SHS exposure effects. Thus, TIMP-1 (p < 0.01) and TIMP-3 (p < 0.05) protein expression were reduced in MYS, MOC and MOS rat hearts. TIMP-2 (p < 0.01) and TIMP-4 (p < 0.05) protein expression were markedly reduced in MOC and MOS rat hearts (Figure 6). In the MYS group, TIMP-1 and TIMP-2 showed no changes when compared with the MYC group.

DISCUSSION

Human cardiac aging generates a complex phenotype. Experimental evidence in animal models indicates attenuation in cardio protective pathways with aging, yet limits myocardial dysfunction information in the aging.18 Some of the age-associated changes in the heart can be reversed, at least partially, by exercise or specific drugs.19 It remains unclear whether aging would result in any definite disease. The changes in the heart throughout life are the result of maturational changes beyond sexual maturity. The age-related changes include controlled myocyte hypertrophy and capillary endothelial cell and interstitial fibroblast hyperplasia.20 The age and disease-dependent alterations in total MMP activity are changes to the myocardial collagen content occurring in cardiac fibrosis. We make separate aging-related changes from those related to disease in this study, and outline their significance for cardiac remodeling. In the aging heart, fibroblasts transition into myofibroblasts and extracellular matrix protein accumulates in the interstitium.21 The histopathologic images show fibrotic remodeling of the left ventricular in aging and SHS exposed hearts (Figure 1). Fibrosis occurs in most injuries and results from changes in the balance between extracellular matrix component synthesis and degradation. Transforming growth factor-β1 (TGF-β1) is a biologically active peptide present in normal cells, including fibroblasts.22 According to the results noted in Figure 2 results, TGF-β1, p-Smad2/3 and CTGF stimulate collagen expression and its appearance in the extracellular matrix in aging and SHS-exposed rats. FGF-2 and UPA may contribute to fibrosis by collagen accumulation, using Masson's trichrome (Figure 2 and Figure 3). However, urokinase overexpression causes accelerated atherosclerosis, coronary artery occlusions and premature death.23 We observed no changes in young rat hearts exposed to SHS. This is possible by simultaneously targeting multiple pathogenic pathways. The matrix metalloproteinase (MMPs) is an endogenous enzyme system responsible for extracellular collagen degradation24 and inhibiting TIMPs expression (Figure 4 and Figure 5). At this time, elevated MMP2 and MMP9 expression occurs during fibrogenesis permitting an immediate metalloproteinase reactivity as soon as TIMP-1, TIMP-2, TIMP-3 and TIMP-4 levels decline (all p < 0.05). With aging-dependent changes in cardiac remodeling, deregulation of MMP2 and MMP9 are believed to contribute to fibrosis and aging.25 These observations sug-
gest that depression of the degradative pathway is partly responsible for age-associated fibrosis. However, MMP 2 and MMP9 activity decrease with aging and increase with exposure to SHS. TIMPs (TIMP-1, TIMP-2, TIMP-3 and TIMP-4) elevation is observed with aging and is consistent with upstream suppression of active and ECM fraction forming MMP2 and MMP9. Interestingly, these results were observed in aging rat hearts exposed to SHS. TIMP-4 expression in the aging heart exposed to SHS, with failing myocardium could lead to enhanced ECM remodeling through loss of MMP-2 leading to cardiac dysfunction.26 In summary, TGFβ1 is a potent TIMPs stimulator including TIMP-1, TIMP-2, TIMP-3 and TIMP-4, and a potent contributor to fibrosis in the aging LV. We have provided evidence indicating that aging suppresses MMPs (MMP 9 and MMP 2) production in the left ventricle.

CONCLUSIONS

Cardiovascular disease is a leading cause of morbidity and mortality, accounting for approximately 30-40% of deaths in the elderly stage. Most of vascular disease during old age occurs arteriosclerosis resulting in stiffening and left ventricular fibrosis. Smoking and SHS will lead to this two main forms serious, especially in old age, but not occur young. Therefore, we suggest that SHS exposure enhances cardiac fibrosis on aging rat hearts.

ABBREVIATIONS

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<tr>
<th>Abbreviation</th>
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<tr>
<td>ECM</td>
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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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