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The role of the mucosa in modulation of evoked responses in the spinal cord injured rat bladder

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Aims: Mounting evidence indicates that a variety of factors released from the urothelium or suburothelium can modulate smooth muscle activity. Although the relationship between the mucosa and smooth muscle has been investigated, little is known about the pathophysiologic changes in detrusor-mucosa interactions in neurogenic bladders. The goal of the study was to determine the impact of the mucosa on evoked responses in spinal cord injured (SCI) bladders.

Methods: Urinary bladders were obtained from 6wk SCI rats or age-matched uninjured controls. Ex vivo isometric tension studies were performed and muscarinic receptor expression was measured in bladder tissue with and without mucosa.

Results: The magnitude and area of nerve evoked responses in SCI tissue with mucosa was higher than without mucosa. The duration and decay time of nerve-evoked responses were longer in SCI than control tissue irrespective of the mucosa. The level of the muscarinic M_2 receptor was decreased in the mucosa of SCI bladders.

Conclusions: Detrusor-mucosa interactions are substantially altered in the neurogenic bladder. After spinal cord injury, an excitatory modulation of smooth muscle contraction by the mucosa emerges, and could be targeted via intravesical treatment in the context of neurogenic bladder dysfunction.

KEYWORDS
detrusor contractility, mucosa, neurogenic bladder, spinal cord injury

1 | INTRODUCTION

Contraction of bladder smooth muscle is regulated in part by neurotransmitters released from intramural varicosities. In addition to neural control, bladder smooth muscle contractile responses may be modulated by a variety of bioactive substances released from the urothelium or suburothelium,[1–3] including ATP, acetylcholine, nitric oxide, prostaglandins, and neuropeptides. These mucosa-derived factors can be released in response to mechanical, chemical, thermal, and hormonal stimulation.

Interaction between the detrusor and mucosa is known to generate or augment spontaneous contractions, and is readily confirmed by the marked reduction in this activity in tissues in

which the mucosa has been removed.[4] This excitatory modulation of bladder function by the mucosa may serve to maintain adequate tone during physiologic bladder filling. Thus, mucosal alterations associated with pathologic conditions may amplify spontaneous activity and promote detrusor overactivity.

In contrast to excitatory detrusor-mucosa interactions in response to stretch or bladder filling, the impact of the mucosa on stimulation-induced responses of bladder smooth muscle is thought to be inhibitory. Removal of the mucosa has been shown to increase the contractile response to various stimuli,[5,6] suggesting an unknown inhibitory factor is released from the mucosa in response to stimulation in normal human, pig, cat, and rodent bladders. Although the inhibitory factor has not been identified, it appears not to be nitric oxide, an adenosine nucleotide, a cyclo-oxygenase product, a catecholamine, or GABA.[1] Nevertheless, epithelium-dependent modulation of smooth muscle tone is a phenomenon recognized in many hollow organs, including the bronchi, trachea, vas deferens, ureter, and uterus.

The complexity of detrusor-mucosal interactions induced by stimulation is underscored by conflicting findings among studies. In contrast to mucosa-dependent inhibition, removal of the mucosa has also been shown to decrease the contractile response to muscarinic receptor stimulation,[7] electrical field stimulation,[8] and ATP,[9] or to have no effect on the response to electrical field stimulation, carbachol,[8] and KCl[10] in the rat bladder. Similarly, in the guinea pig, the presence of the mucosa did not affect (or slightly increased) the response to histamine, bethanechol, KCl,[11] and electrical stimulation.[12] Moreover, a non-adrenergic, non-nitrergic, non-prostanoid relaxant agent that does not originate from the mucosa was reportedly released by stimulation of muscarinic receptors in the detrusor, suggesting an inhibitory factor may also be produced by bladder tissue during contraction.[13]

Evidence abounds that the bladder mucosa provides an abundant source of key molecules and mediators having the potential to modulate bladder smooth muscle responsiveness. However, the conditions under which mucosa-derived factor-(s) exert an inhibitory or stimulatory effect on bladder smooth muscle function is unclear. Moreover, the impact of the mucosa on contractile responses under pathological conditions has not been studied in depth. Damage to the urothelium and consequent loss of detrusor-mucosal interactions may alter the level and range of these mediators and modify the reactivity of bladder smooth muscle. Uncovering altered mucosal contributions to detrusor-mucosal interactions may guide the management of pathological conditions and support the use of intravesical delivery methods to more effectively target the principal source of bladder dysfunction while minimizing side effects of systemic approaches. Following spinal cord injury (SCI), the mucosa undergoes significant remodeling, including urothelial proliferation and regeneration at different timepoints.[14,15] Thus, the aim of this study was to determine the effect of the mucosa on evoked contractile responses in SCI bladder tissue compared with control tissue.

2 | MATERIALS AND METHODS

2.1 | Ethical approval

These studies and methods were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experimental protocols were approved by the Animal Care and Use Committee of Boston Children's Hospital (protocol #16-08-3256R).

2.2 | Creation of spinal cord injury

Male Sprague Dawley rats (7 weeks, ~250 g, Charles River Laboratories, Wilmington, MA) were subjected to mid-thoracic spinal cord transection essentially as described.[16] Briefly, under isoflurane anesthesia a dorsal midline incision was made over the thoracic spinal cord. Superficial and deep muscle layers were incised in the midline to expose the spine and a laminectomy was performed. The dura was transected sharply and the cord was severed at T8. Gelfoam (Ethicon™) was placed between the two cut ends of the spinal cord to aid in haemostasis; the dura was not closed. The paraspinal muscles and skin were closed in two separate layers. Post-operative pain was managed with meloxicam analgesia (5 mg/mL, subcutaneously every 24 h for 3 days). Rats received Baytril antibiotic prophylaxis (100 mg/mL at 7.5 mg/kg, s.c) during the post-operative period. During the period of spinal shock, characterized by bladder areflexia, bladders were emptied every 12 h by manual expression until reflex voiding returned. This period lasted for 10 days to 2 weeks. Rats were monitored and weighed on a daily basis. A cohort of age-matched uninjured rats was used as controls (Ctrl).

2.3 | Ex vivo contractility studies

Six weeks after spinal cord transection, bladders from SCI animals ($n = 34$) were harvested following euthanasia via CO_2 inhalation and placed in ice-cold Kreb's buffer (NaCl 120 mM: KCl 5.9 mM; NaHCO$_3$ 25 mM; Na$_2$H$_2$PO$_4$ 1.2 mM; MgCl.6H$_2$O 1.2 mM; CaCl$_2$ 2.5 mM; dextrose 11.5 mM) for ex vivo contractility analyses. Bladders from uninjured animals ($n = 16$) were similarly harvested at 13 weeks of age. Tissues not required for functional assays were flash frozen and stored at $-80\,°C$ for molecular analysis. Bladders from both control and SCI animals were carefully cut into longitudinal strips. From some of the strips, the mucosa was detached as a continuous sheet by microdissection to ensure its complete removal. Bladder tissue with or without intact mucosa was mounted in tissue chambers maintained at

37°C and bubbled with a mixture of 95% O_2 and 5% CO_2. Bladder tissue was attached to a force transducer (Grass Instruments) and stretched to 1.5 grams of passive force. Following an equilibration period of an hour, electrical field stimulation (EFS) was carried out over a range of frequencies (1–64 Hz, 20 V, 0.5 ms pulse widths, 10 s duration). At each frequency of stimulation, the neurally-evoked contractile response was characterized by the following parameters using LabChart Pro 8: peak amplitude of the contraction, area under the force response curve, slope of the ascending phase of contraction (between 10% and 90% of the peak), width of the response at 50% of the peak, and the time constant (tau) of the exponential decay curve fitted to the descending phase of the contraction (Figure 1). In addition, contractile responses to carbachol (1–10 μM; Sigma–Aldrich, St. Louis, MO) and α,β-methylene-ATP (10 μM; Sigma–Aldrich) were also measured. Conditioned signals from force transducers were continuously acquired at 50 Hz by a 16-channel analog-to-digital converter (DataQ, DI-720) and recorded to disk using Windaq data acquisition software.

2.4 | Real time RT-PCR

RNA was isolated from bladder tissue without mucosa and from mucosa alone ($n = 4$ controls, $n = 4$ SCI) using TRIzol reagent along with the RNeasy MiniKit (Qiagen, Frederick, MD). RNA was reverse-transcribed using a high-capacity cDNA synthesis kit (Applied Biosystems-Life Technologies, Foster City, CA) according to the manufacturer's instructions. cDNAs were amplified using TaqMan gene-specific assays for M_2 and M_3 muscarinic receptor subtypes, and relative mRNA levels were determined following normalization to GAPDH.
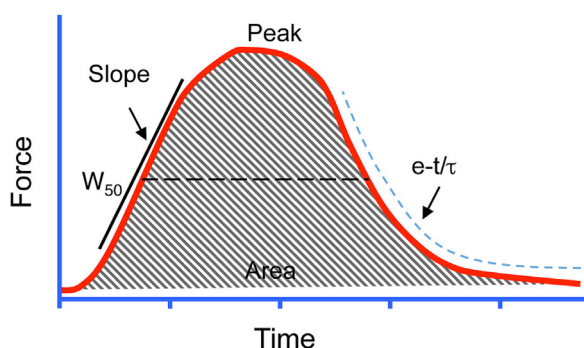

FIGURE 1 Diagrammatic representation of the parameters calculated from the EFS-evoked responses. The peak was measured as the maximum amplitude of force and normalized by cross-sectional area of the tissue. Hatched region shows area under the force curve. Slope (solid black line) was calculated from the tangent to the ascending phase of contraction. Half-width (W_{50}, dashed line) is the duration of the contraction at 50% of peak force. An exponential decay function (blue dashed curve) was fit to the descending phase of the contraction (after termination of stimulation) and its time constant, τ, was used to indicate the rapidity to baseline

2.5 | Immunoblot analysis

Bladder tissues from both SCI and age-matched control (Ctrl) rats were used to generate protein lysates of detrusor either with or without mucosa. Protein concentrations were determined by the bicinchoninic acid protein assay (BCA). Equal amounts of protein lysate from Ctrl and SCI were loaded onto a 4-12% SDS bis-tris polyacrylamide gel and resolved by electrophoresis. Proteins were then transferred to nitrocellulose membrane (0.2 μm pore, Invitrogen, Carlsbad, CA). Non-specific binding was inhibited by incubating membranes in TBST with 5% dry milk for 1 h. Membranes were incubated overnight at 4°C with the following primary antibodies: rabbit anti-muscarinic M3 receptor (1:250), rat anti-muscarinic M2 receptor (1:250), mouse anti-β-actin (1:2500, Santa Cruz Biotechnology, Dallas, TX), and rabbit anti-purinergic P2×1 receptor (1:1000, Alomone Labs, Jerusalem, Israel). Membranes were then washed extensively with TBST and incubated for 1 h at room temperature with HRP conjugated anti-rabbit, anti-rat, or anti-mouse secondary antibodies (Invitrogen). After incubating the membrane with a chemiluminescence substrate, immunoreactive bands were visualized using a digital imager (Amersham 600). The intensity of each band was quantified from digital images using Image Quant Software, normalized by the intensity of internal control (β-actin), and compared between Ctrl and SCI samples.

2.6 | Statistical analysis

Functional data related to the amplitude of agonist or EFS-induced contractions were expressed as force (mN) normalized by tissue cross-sectional area and presented as mean ± SEM. Differences in responses among SCI and control tissue with or without mucosa were determined by analysis of variance followed by Holm-Sidak test. RT-PCR data were analyzed using the Mann–Whitney U test. Differences in levels of receptor expression between SCI and control samples were determined by t-test. $P \leq 0.05$ was considered statistically significant.

3 | RESULTS

The body weight of SCI rats (403.44 ± 10.34 g, $n = 41$) was not different from control animals (371.06 ± 19.19 g, $n = 16$). However, the bladder-to-body weight ratios were significantly higher in SCI rats versus uninjured controls (0.545 ± 0.033 vs 0.244 ± 0.013 mg/g).

3.1 | Mucosa exerts an excitatory effect in SCI bladder tissue

The magnitude of the frequency-response curve in control tissue with intact mucosa was not significantly different than mucosa-denuded control tissue (Figure 2A). In contrast, the
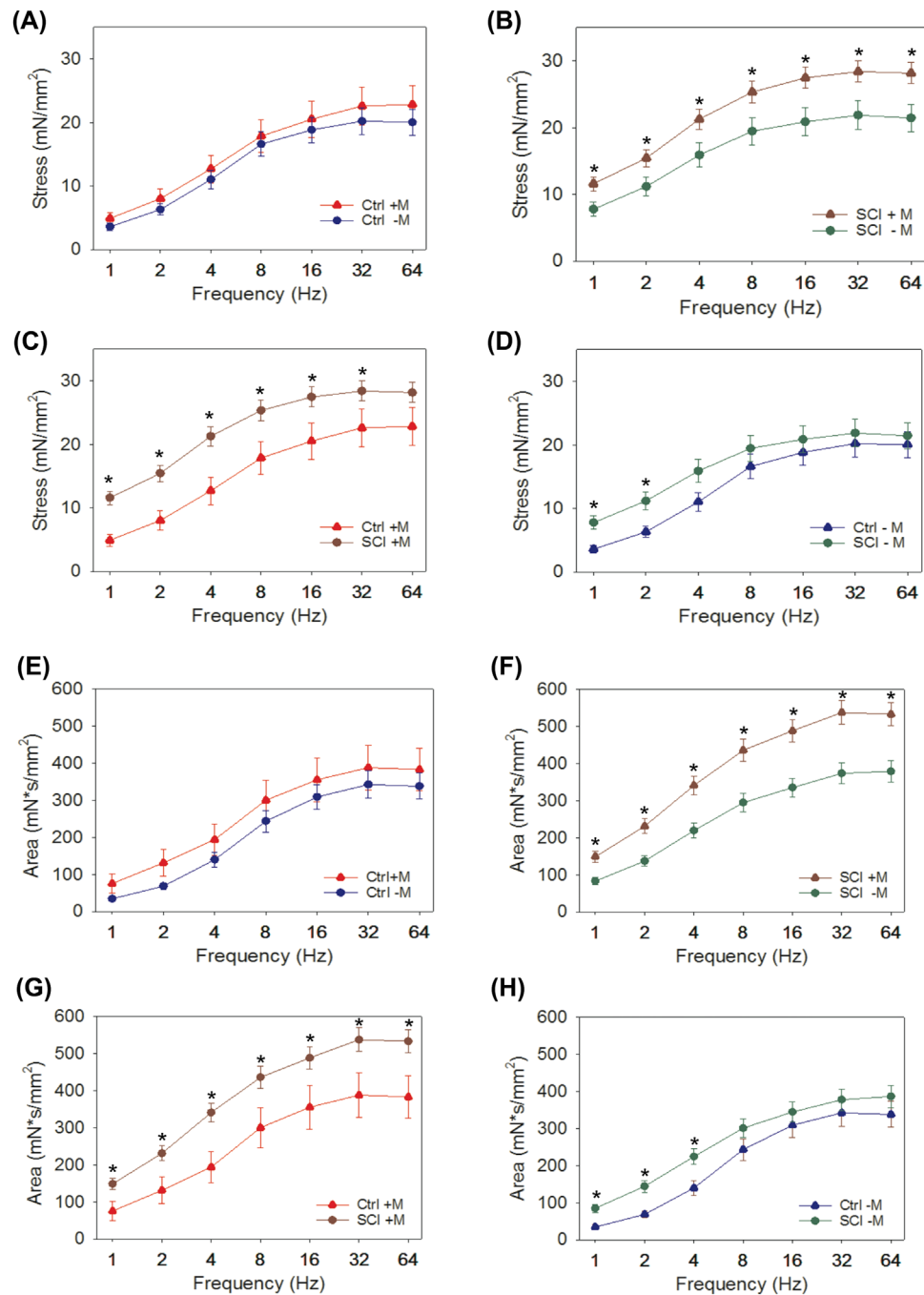
FIGURE 2 Mucosa-dependent excitatory effect in SCI bladder tissue. The peak of the frequency-response curve was compared in (A) Ctrl bladder strips with (red triangle, +M, $n = 16$) and without mucosa (blue circle, −M, $n = 16$) and (B) SCI with (brown triangle, +M, $n = 34$) and without mucosa (green circle, −M, $n = 34$). Removal of mucosa did not alter responses significantly in Ctrl tissues (A), whereas the presence of mucosa enhanced the contractile response in SCI tissues (*$P < 0.05$). Data from Ctrl and SCI strips with (C, control [red circle] and SCI [brown triangle]) or without (D, control [blue triangle] and SCI [green circle]) mucosa were re-plotted to enable easier comparison of responses without (Ctrl) or with (SCI) injury, in the presence or absence of mucosa, respectively. E–H, Effect of mucosa on the area of the frequency-response curves in Ctrl and SCI tissues. E, Removal of the mucosa had no significant effect on Ctrl tissues. F, The area under the curve of SCI strips with intact mucosa (brown triangle, $n = 34$) was significantly higher than that of strips without mucosa (green circle, $n = 34$)(*$P < 0.05$). G, The area under the curve in tissues with intact mucosa was significantly higher in SCI than Ctrl at all frequencies. H, In tissue with denuded mucosa the area under the curve in SCI was significantly higher at 1 and 2 Hz compared to Ctrl (*$P < 0.05$)

response to EFS in SCI bladder tissue with intact mucosa was significantly higher ($P < 0.05$) at all frequencies compared to SCI tissue in which mucosa was removed (Figure 2B). Thus, in neurogenic bladder the mucosa exerts an excitatory effect on smooth muscle activity. With an intact mucosa, the maximum amplitudes of EFS responses at frequencies <64 Hz were significantly higher in SCI tissue than controls (Figure 2C). In tissues in which the mucosa had been removed however, the response to EFS in SCI rats was greater than controls only at low frequencies of stimulation (<4 Hz) (Figure 2D).

The area under the curve for SCI bladder was significantly higher ($P < 0.05$) in tissue with intact mucosa than without mucosa (Figure 2F). In contrast, the area under the curve in control tissue with intact mucosa was not significantly different from tissue without mucosa (Figure 2E). In tissue with intact mucosa the area under the curve was significantly higher in SCI than control at all frequencies tested (Figure 2G). With denuded mucosa, the area in SCI tissue was augmented compared to control only at lower frequencies of stimulation (Figure 2H).

The widths of contractions at 50% of the peak were significantly higher at all frequencies in SCI compared to control tissue (Figures 3C and 3D), independent of the presence of mucosa (Figures 3A and 3B). The time constant, tau (τ), a measure of the relaxation phase of the evoked response, was significantly higher at all frequencies in SCI bladder tissues compared to control tissues, (Figures 3G and 3H) but was not affected by the mucosa in either group (Figures 3E and 3F).

The slope of the ascending phase of the nerve-evoked response was significantly higher with an intact compared to a denuded mucosa in both control and SCI bladders (Figures 4A and 4B). Compared to control tissue, the slope was lower in SCI bladders at higher frequencies, independent of the presence of the mucosa (Figures 4C and 4D).

3.2 | Mucosa does not affect post-junctional evoked responses in SCI bladder tissue

The dose response to the cholinergic agonist carbachol was also determined in bladder tissue with and without mucosa from control ($n = 15$) and SCI ($n = 15$). The presence of the mucosa did not affect the dose response curve to carbachol in either control or SCI tissue (Figures 5A and 5B). With an intact mucosa, the response to carbachol in control tissue was not different from that in SCI tissue (Figure 5C). However, in tissue without mucosa, the response to carbachol was significantly higher in SCI tissue at doses between 0.03 and 1 μM compared to control tissue and the half maximal effective concentration (EC_{50}) in SCI tissue (340 ± 75.7 nM) was significantly lower than in control tissue (916 ± 19.8 nM) (Figure 5D). This difference in EC_{50} between SCI and

controls was not observed in strips in which mucosa was intact. In tissue with intact mucosa, contractions generated in response to the purinergic agonist α,β-meATP in control tissues were not different from those recorded in SCI tissues. Removal of mucosa decreased the response to α,β-meATP in both groups, but neither reached statistical significance (Figure 5E).

To explore potential differences in neurotransmitter receptor expression, we analyzed tissues from Ctrl and SCI rats using semi-quantitative real time RT-PCR and immuno-blotting. Analysis of M_2 and M_3 receptor mRNA levels revealed differences between control and SCI detrusor and mucosa. Both M_2 and M_3 levels were significantly lower in the SCI mucosa versus control mucosa, whereas no significant difference in levels of either receptor was observed in detrusor (Figure 6A). In mucosa-denuded tissue, the expression of muscarinic receptors M_2 and M_3 detected by immunoblot was not different between control and SCI animals. However, in bladder tissue with intact mucosa, the level of M2 receptor expression was significantly decreased in SCI rats relative to that in control animals (Figures 6B and 6C), while M_3 receptor expression was unchanged. The level of P_2X_1 receptor expression in SCI tissue was not different from control samples, irrespective of the mucosa (Figures 6C and 6D).

4 | DISCUSSION

Epithelium-dependent modulation of underlying smooth muscle has been demonstrated in a number of organs, including the bladder. Factor(s) generated in the urothelium or suburothelium may activate the production or release of mediators in the suburothelium to alter smooth muscle function. Our findings indicate that the mucosa does not inhibit contractile responses to evoked stimulation in the rat with an intact neuroaxis. However, the nominal excitatory effect of the mucosa on bladder smooth muscle reactivity demonstrated in normal bladder was remarkably enhanced after spinal cord injury. The aggregated functional response may depend on the balance between the effects of mucosa-derived inhibitory and excitatory factors, which are determined by the specific collection of receptors, enzymes and cell types that are engaged. The magnitude of mucosal dependency is likely shaped by a variety of factors including species, gender, age, and region within the bladder, as well as diseases that alter mucosal structure and function.

Distinct changes in the urothelium are known to occur after SCI with the regions of incomplete differentiated urothelium evident even after 28 days following SCI.[14,15] Analysis of histopathological samples from SCI patients have revealed significant changes to the mucosa of the bladder.[17] In addition, the unique intrinsic activity that is observed after
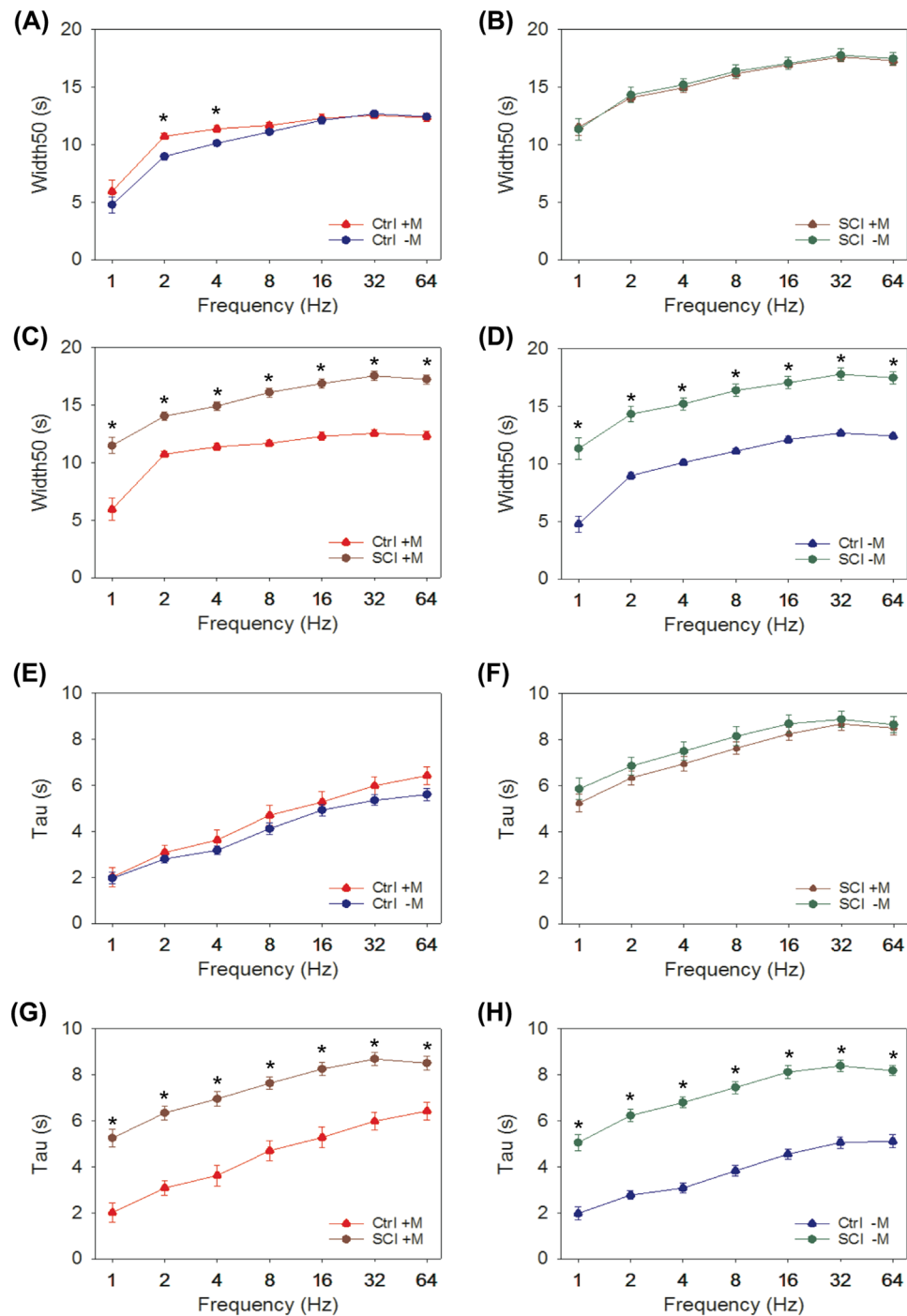
FIGURE 3 Mucosa-independent changes in evoked responses following SCI. Removal of mucosa did not alter the half-width time (duration of 50% of peak contraction) of the frequency-response curves, in either (A) Ctrl or (B) SCI tissues. However, the half-width times were significantly higher at all frequencies in SCI compared to Ctrl in tissues with (C) and without (D) mucosa (*$P < 0.05$). Removal of mucosa did not alter the time constant (τ), in either Ctrl (E) or SCI (F) tissues. The time constant was significantly higher at all frequencies in SCI compared to Ctrl in tissues with (G) and without (H) mucosa (*$P < 0.05$)

SCI has been described to be of urothelial origin.[18] Previous reports from our group and others described an increase in bladder spontaneous activity in tissue from rats with SCI versus that from non-injured controls.[16,19] In addition, a decrease in the amplitude of SA upon removal of mucosa was noted, consistent with the idea that the mucosa can modulate

detrusor activity. In the present study, evaluation of the mucosa from SCI bladders was extended to its effect on contractile responses evoked by either nerve stimulation or exogenous administration of excitatory agonists.

Following SCI, the kinetics and time course of the mechanical response to field stimulation were dramatically
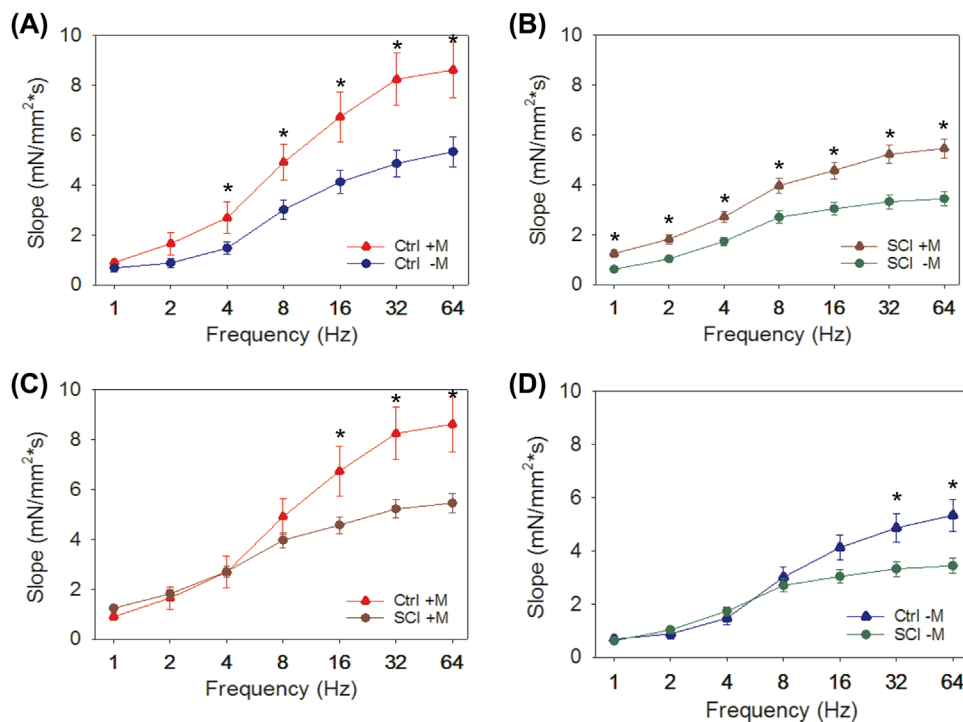
FIGURE 4 Mucosa- and SCI-dependent changes in evoked responses. Following removal of the mucosa, the slope of the ascending phase of the contraction in frequency-response curves was significantly lower in both Ctrl (A) and SCI (B) compared to tissues in which mucosa was intact (*$P < 0.05$). In both the presence (C) or absence (D) of mucosa, the slope was greater at higher frequencies in Ctrl compared to SCI tissue (*$P < 0.05$)

altered. A number of parameters derived from the force response curves were used to characterize the distinctive changes in neurally-evoked contractions, and the impact of the mucosa on modulating these parameters were investigated. Irrespective of the mucosa, the half-width time of the contraction and the time constant of the relaxation phase were significantly increased after SCI. These results are consistent with findings from human hyperreflexic bladders which showed a protracted half-width and an increased time to peak force generated in response to electrical stimulation[20] and indicate that SCI profoundly alters the ability of bladder to relax once activated. Whether this prolonged response is neurogenically mediated or caused by changes in electrophysiological properties of smooth muscle cells or altered viscoelastic behavior of bladder tissue warrants further investigation. Nevertheless, the prolonged half-width time and increased relaxation time constant reflect an increase in the total duration of contraction. As bladder contractility is defined not only by the magnitude and rate of rise of the isometric contractile response, and but also by the duration of contraction,[21] our findings indicate that intrinsic bladder contractility is preserved in response to functional obstruction and axonal degeneration after SCI by an increased contraction duration.

We found that the mucosa significantly enhanced the magnitude and area of the EFS induced response in SCI

bladder tissue but not in control tissue. Moreover, with an intact mucosa, peak and area responses in SCI tissue were significantly higher than mucosa-intact control bladders, suggesting that the mucosa exerts an excitatory effect on smooth muscle contraction in neurogenic bladders. Although a decreased response to neural stimulation has been reported in human tissue from neurogenic bladders,[20] an increased sensitivity to field stimulation has also been described,[22] similar to our findings in the SCI rat bladder. Though the mechanism(s) contributing to the enhanced response were not explicitly examined, a loss of inhibitory neuromodulation, or decreased autoinhibition of cholinergic transmission are potential factors. In patients with neurogenic bladder, a reduced number of VIP (vasoactive intestinal peptide)[22,23] and neuropeptide Y immunoreactive nerves[24] was proposed as a basis for increased reactivity to neurally-evoked stimulation.[22] In addition, pre-junctional M_2 receptors have been shown to decrease contractions in the rat bladder.[25] Our findings of enhanced contractile response to nerve mediated stimulation in association with reduced mucosal expression of M_2 receptors in spinal transected animals is consistent with increased release of acetylcholine due to loss of inhibitory autoreceptors conceivably in suburothelial nerve fibers. Although expression of both muscarinic receptors was reduced in SCI mucosa at the mRNA level compared to control, only M_2 receptor protein levels were reduced,
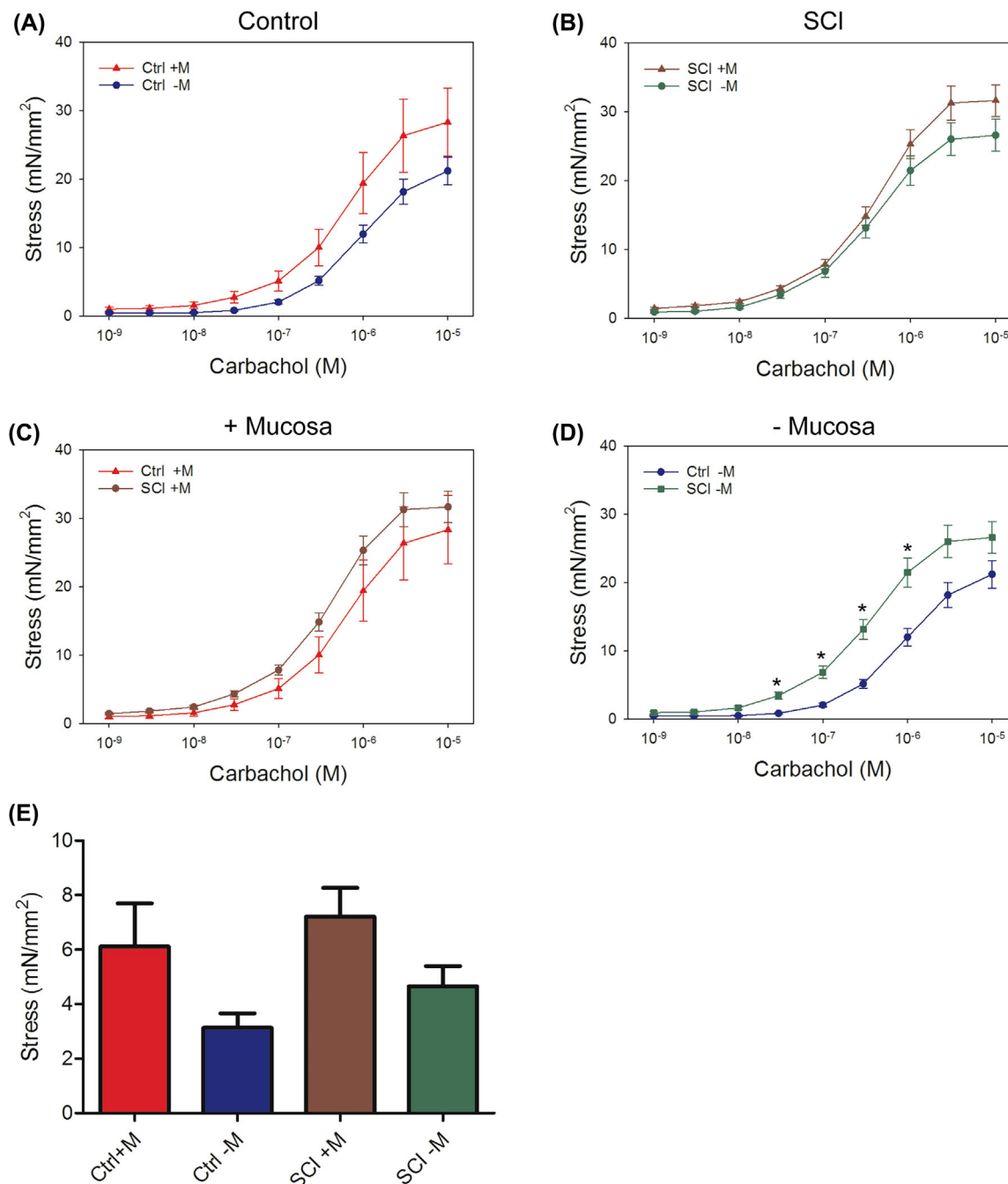
FIGURE 5 Effect of mucosa and SCI on agonist-induced contractions. Dose response curves for carbachol (1-10 µM) in (A) Ctrl with (red triangle, +M, $n = 15$) and without mucosa (blue circles, −M, $n = 15$) and (B) SCI with (brown triangle, +M, $n = 15$) and without mucosa (green circle, −M, $n = 15$); Comparison of dose-response curves from Ctrl and SCI bladders with (C) and without (D) mucosa. The response of SCI tissues without mucosa to carbachol is significantly greater than that of Ctrl tissues without mucosa (*$P < 0.05$) (E) Contractile response to α,β me-ATP (10 µM) in control ($n = 11$) and SCI ($n = 11$) with and without mucosa. Data presented as mean ± SEM

consistent with the lack of correlation between protein and transcript levels previously reported for the M_3 receptor.[26]

In the bladder, ATP is co-released with acetylcholine from parasympathetic nerve terminals and its proportional effect on the contractile response is greater at lower stimulation frequencies.[27] Therefore, the enhanced contractile response to nerve stimulation predominately at low frequency could be attributed to increased sensitivity to ATP. However, apart from than the increased mechanically induced release of ATP from the urothelium[28] after SCI, altered neurogenic ATP release or increased atropine-resistant contractions have not been reported in SCI bladders. Moreover, the expression of P2×1 receptor and contractile response to ATP were not altered by SCI in this study.
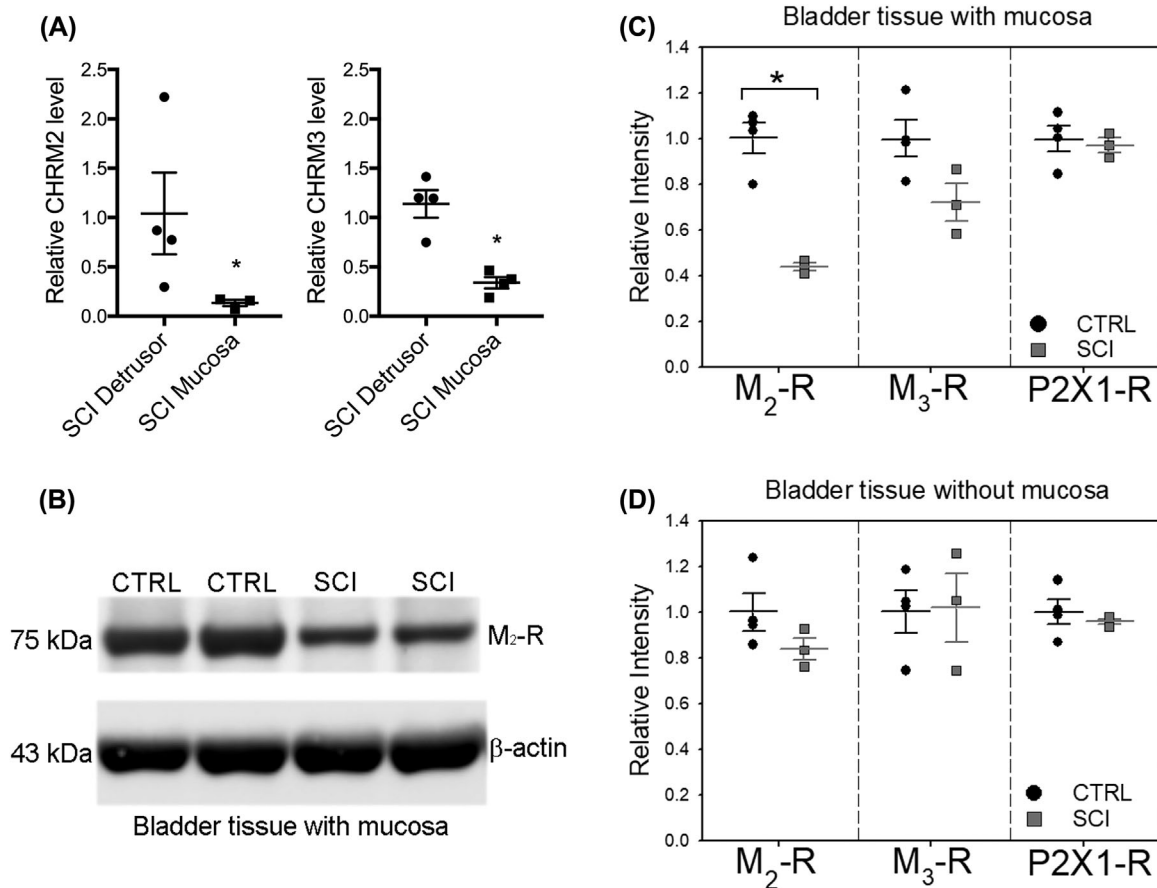
FIGURE 6 Determination of neurotransmitter receptor levels. A, RT-PCR analysis of gene expression of M_2 receptor (CHRM2) (left panel) or M_3 receptor (CHRM3) (right panel) in the detrusor or mucosa of SCI bladders. Expression levels in SCI tissues are reported relative to the respective tissue from Ctrl (detrusor or mucosa, relative level = 1), and data are presented as mean ± SEM. *$P < 0.05$, compared to respective control tissue. B, Representative immunoblot for M2-R and β-actin in bladder tissue with mucosa from Ctrl and SCI rats. Quantification of levels for M2-R, M3-R, and P2×1-R in Ctrl or SCI tissues with (C) or without (D) mucosa revealed a significant decrease in M2-R in detrusor strips with mucosa intact following SCI ($n = 3$) compared to Ctrl ($n = 4$). Band intensities were normalized by the internal control β-actin. Expression levels in SCI tissues were reported relative to control tissue. Data are presented as mean ± SEM. *$P < 0.05$

Alternatively, facilitation of cholinergic neurotransmission may underlie our findings that, in the absence of the mucosa, the magnitude, and integrated contractile responses of SCI tissue were augmented particularly at lower stimulation frequency. In rat neurogenic bladder, pre-junctional M_1 receptor-mediated facilitation of acetylcholine release, which normally occurs only at high stimulation frequency, emerges at lower frequencies of stimulation after SCI.[29]

The mechanical response to nerve stimulation consists of an initial rapid transient phase that is sensitive to purinergic desensitization but relatively resistant to atropine, and a late phase that is sensitive to atropine but resistant to purinergic desensitization.[30] Therefore, the sharp rise of force reflects the rapid actions of nerve evoked release of ATP on ionotropic P2X receptors and smooth muscle membrane depolarization.[31] We found that the slope of the rising phase of the contraction was enhanced in both control and SCI tissue with an intact mucosa. The mucosa is a major source of ATP that can be released, particularly from the urothelium, to act on nerves and

interstitial cells in the suburothelium and underlying smooth muscle.[2] This mechanism may contribute to the mucosa-dependent acceleration of nerve evoked force generation. The release of endogenous ATP can be induced by activation of urothelial muscarinic receptors in sufficient amounts to cause contraction.[2] Thus, a reduction in muscarinic receptor expression in the mucosa after SCI could attenuate the ATP mediated increase in the slope of contraction.

To determine whether the effect of the mucosa was specific to neurally mediated contractile responses, we compared post-junctional contractions induced by exogenous administration of cholinergic and purinergic agonists in Ctrl and SCI tissue in the presence or absence of the mucosa. The post-junctional cholinergic and purinergic contractile responses were not affected by the mucosa in SCI or neurally-intact animals. With an intact mucosa, the contractile response to carbachol after SCI was not different from control.

The sensitivity to carbachol in the absence of the mucosa, however, was significantly increased in SCI tissue

compared to Ctrl. In patients with neurogenic bladder, both M2 and M3 muscarinic receptors appear to mediate bladder contraction,[32] in contrast to normal bladders in which only the M3 receptor is responsible for contraction. A shift from M3- to M2-mediated contractions, along with increased M2 receptor density, was also demonstrated in rats with denervated bladders and in SCI animals that did not void spontaneously.[33] In SCI rats that were able to void spontaneously however, M3-mediated contractions occurred together with unchanged M2 receptor density, but decreased M3 receptor density.[33] Despite an increased M2:M3 ratio in these bladders, the sensitivity to muscarinic agonists was not altered. Although the lack of M2 receptor changes is similar to our findings in male SCI rats with reflex voiding, directional differences between studies in M3 receptor expression and EC_{50} may be related to gender. A previous study showed that aging changed the contractile phenotype from M3- to M2 receptor- mediated responses in male rats but not in females.[26] While changes in muscarinic receptor expression do not appear to underlie the enhanced carbachol sensitivity found in our model, an alteration in contractile phenotype potentially due to differential coupling of M2 receptors to G proteins or interaction with PKC cannot be ruled out. Other post-junctional mechanisms may contribute to increased smooth muscle responsiveness. Structural changes in smooth muscle cell profiles described as abnormal protrusion junctions and ultraclose abutments observed in patients with neurogenic bladder[34] have been suggested to augment coupling between smooth muscle cells after spinal cord injury. Moreover, an increased expression of connexin 43 detected in patients with neurogenic bladders[35] is consistent with enhanced electrical coupling among bladder smooth muscle cells. Altered calcium sensitivity or ion channel activity due to increased RhoA/Rho-kinase signaling[36] or decreased calcium-activated potassium channels[37] reported after SCI may also contribute to enhanced carbachol sensitivity.

5 | CONCLUSIONS

Our data show that pre-junctional and post- receptor mechanisms contribute to enhanced evoked responses after SCI. SCI increases the nerve-mediated excitatory response by altering the impact of the mucosa on force generation. This may suggest that the mucosa releases an excitatory factor that influences smooth muscle evoked responses. Investigation of the identity of such factors warrants further investigation.

CONFLICTS OF INTEREST

The authors declare there are no financial conflicts of interest to disclose.

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