Impairment of diaphragm muscle force and neuromuscular transmission after normothermic cardiopulmonary bypass: effect of low-dose inhaled CO

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THE USE OF CARDIOPULMONARY bypass (CPB) is crucial for the success of many cardiac operations. However, CPB exerts a robust systemic response involving cytokine and other inflammatory mediators (7, 9, 33, 38). Usual strategies to mitigate the impact of CPB-induced systemic responses on the outcome of cardiac surgical patients include redesigned circuits and components, novel surgical techniques, and pharmacological agents (33). Recently, low-dose, inhaled carbon monoxide (CO) has been studied in animal models of cell injury (6, 15, 17, 18, 29, 37, 48, 50). In addition, several lines of evidence suggest that CO is an important chemical signal that modulates neurotransmission (24). Thus, we hypothesized that 1) CPB exerts deleterious effects on rat diaphragm muscle force and neuromuscular transmission, and 2) low-dose CO treatment during CPB will mitigate these effects.

MATERIALS AND METHODS

Animals. The study’s protocol was approved by the Institutional Animal Care and Use Committee, and all procedures were performed in accordance to the institutional and American Physiological Society’s Guiding Principles in the Care and Use of Animals, national guidelines for compassionate and humane animal care. Eighteen male Sprague-Dawley rats weighing ~450 g were randomly assigned to three groups: one sham-control and two CPB groups. The sham-control group (Sham-CTL) underwent mechanical ventilation, as well as tracheal and vascular cannulation without CPB (n = 6 animals). Two experimental groups underwent CPB for 1 h, during which one group was exposed to air (CPB-Air group; n = 6 animals), and the other group was exposed to CO (250 ppm in air; CPB-CO group; n = 6 animals). CPB was followed by a 90-min recovery period. Animals in the CPB-CO group were only exposed to CO during CPB.

Surgical procedures. All surgical procedures were performed under adequate anesthesia achieved using intramuscular ketamine (90 mg/kg im) and xylazine (10 mg/kg im), and maintained throughout the procedure with additional doses as needed. The right femoral artery was cannulated and used for continuous direct arterial pressure monitoring and as a medication port. The right internal jugular vein and left carotid artery were used as the venous and arterial cannulation sites for CPB, respectively. Tracheostomy was performed via midline incision, and the trachea was cannulated with a 14-gauge intravenous catheter. A volume-cycled rodent ventilator (model no. 683; Harvard Apparatus, South Natick, MA) was set to deliver a tidal volume of 6
ml/kg and a respiratory rate of 60/min initially was titrated according to blood-gas results. Heparin (500 U/kg) was administered via the femoral artery catheter, and the rat was placed on CPB.

Cardiopulmonary bypass. A modified Plexiglas-encased oxygenator with a static prime volume of 4.5 ± 0.5 ml was custom-built based on the previously reported model developed by You et al. (49). The oxygenator comprised a polypropylene sheet membrane material (surface area: 24 in²; Sorin Group, Milan, Italy), a 30-ml plastic syringe barrel used as a reservoir, and a stainless-steel, heat-exchange device. The reservoir was sealed, and vacuum was applied as needed (20–40 mmHg). The total reservoir volume was 6 ml with ~2 ml as the operating level during CPB. Rats were kept normothermic during CPB using the heat exchanger.

Beating-heart CPB was applied using a miniature extracorporeal circuit composed of nonblood prime. Flows were maintained at 70–80 ml-kg⁻¹ min⁻¹ with a minipump (Minntech Renal Systems, Minneapolis, MN). Mean arterial pressures were maintained at 50–60 mmHg. All the postoxygenator blood flow was directed through an inline blood gas monitoring cell (CDI 500; Terumo, Tokyo, Japan), providing continuous blood-gas analysis, including PaO₂, PaCO₂, pH, temperature, and potassium level. The monitor was calibrated upon initiation of CPB. The circuit was primed with a total of 13 ml of a mixture containing 25 ml of a Rees-Simpson solution of the following composition (in mM): 135 NaCl, 5 KCl, 2 CaCl₂, 1 MgSO₄, 120 Cl⁻, 25 HCO₃⁻, pH 7.4. The central tendon was attached to a force transducer (model 6350; Cambridge Technology, Cambridge, MA), while the rib insertion was clamped with a micro-manipulator. Direct muscle stimulation (1-ms monophasic rectangular pulses via plate electrodes) was used to adjust muscle length until a stable baseline was initiated. Every 15 s, direct muscle stimulation (via plate electrodes; 1-ms supramaximal pulses at 40 Hz in 330-ms trains) was superimposed. The relative contribution of neuromuscular transmission failure to muscle fatique, i.e., NMTF, was estimated by the equation: $NMTF = (F - MF)/(1 - MF)$, where $F$ is the percent decrement in force during repetitive nerve stimulation, and $MF$ is the percent force decrement during direct muscle stimulation (1, 25, 35).

Muscle force and neuromuscular transmission measurements. The procedures for measuring DIAM contractile properties and neuromuscular transmission have been described previously (25, 35). Briefly, for isometric DIAM force measurements, midcaudal DIAM-phrenic nerve preparations (3–4 mm wide) were placed in a vertical organ bath containing warm (26℃) oxygenated (95% O₂:5% CO₂) Rees-Simpson solution of the following composition (in mM): 135 NaCl, 5 KCl, 2 CaCl₂, 1 MgSO₄, 120 Cl⁻, 25 HCO₃⁻, pH 7.4. The central tendon was attached to a force transducer (model 6350; Cambridge Technology, Cambridge, MA), while the rib insertion was clamped with a micro-manipulator. Direct muscle stimulation (1-ms monophasic rectangular pulses via plate electrodes) was used to adjust muscle length until maximal isometric twitch force ($P_t$) responses were obtained (optimal length, $L_o$). Maximum tetanic force ($P_o$) was determined during stimulation trains at 40 Hz for 1 s. Previous studies in the adult rat DIAM showed that maximal tetanic force is elicited at this frequency without resulting in muscle fatigue (1, 25, 27, 35). Evoked isometric force responses were displayed on a storage oscilloscope and recorded with customized software (LabView 8.2; National Instruments, Austin, TX) running on a personal computer.

Both $P_t$ and $P_o$ were determined for each DIAM segment after normalization for estimated cross-sectional area (CSA) of the muscle [$CSA = muscle weight (in g)/1.056 g/cm^3 \times L_o$, (in cm)], where 1.056 g/cm³ is the density of muscle tissue. Wet weight of each muscle strip was determined after removal of any Rees-Simpson solution by blotting and removal of attached central tendon and ribs.

Muscle fatigue resistance was assessed after repetitive stimulation at 40 Hz in trains of 330-ms duration repeated once each second using separate DIAM segments, as previously described (1, 25, 27, 35). The muscular fatigue index (MFI) was calculated as the ratio of force generated after 2 min of stimulation to the initial force.

To measure the rate of neuromuscular transmission failure (NMTF), the phrenic nerve was stimulated (0.2-ms pulse at supramaximal intensity) using a suction electrode at 40 Hz in 330-ms duration trains repeated each second for a 2-min period. Every 15 s, direct muscle stimulation (via plate electrodes; 1-ms supramaximal pulses at 40 Hz in 330-ms trains) was superimposed. The relative contribution of neuromuscular transmission failure to muscle fatigue, i.e., NMTF, was estimated by the equation: $NMTF = (F - MF)/(1 - MF)$, where $F$ is the percent decrement in force during repetitive nerve stimulation, and $MF$ is the percent force decrement during direct muscle stimulation (1, 25, 35).

Statistical analyses. All statistical evaluations were performed using standard statistical software (JMP 5.1.2; SAS Institute, Cary, NC). Data are reported as means ± SE unless otherwise specified. On the basis of our previous studies (25, 27, 35), we estimated that a sample size of $n = 6$ animals per group provides adequate statistical power (i.e., 80% power) to detect an effective size of 1.6 SD units between treatment conditions when using one-way ANOVA with 0.05 two-sided significance level. Statistical analyses were conducted using repeated measures ANOVA or MANOVA (for time-dependent measurements). When appropriate, post hoc group differences were assessed using the Tukey-Kramer honestly significant difference test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Initial body weights were similar across experimental groups (Sham-CTL: 433 ± 12 g; CPB-Air: 442 ± 17 g; CPB-CO: 456 ± 6 g). Baseline characteristics, hemodynamic variables, and blood-gas analyses were also similar between all experimental groups (Table 1). As expected, carboxyhemoglobin was only

Table 1. Hemodynamic variables and blood gas analyses at baseline and during recovery following cardiopulmonary bypass

<table>
<thead>
<tr>
<th></th>
<th>Sham-CTL ($n = 6$)</th>
<th>CPB-Air ($n = 6$)</th>
<th>CPB-CO ($n = 6$)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Recovery</td>
<td>Baseline</td>
</tr>
<tr>
<td>Arterial pressure, mmHg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>systolic</td>
<td>110 ± 4</td>
<td>106 ± 5</td>
<td>115 ± 6</td>
</tr>
<tr>
<td>diastolic</td>
<td>72 ± 2</td>
<td>66 ± 3</td>
<td>76 ± 5</td>
</tr>
<tr>
<td>mean</td>
<td>85 ± 2</td>
<td>79 ± 2</td>
<td>90 ± 6</td>
</tr>
<tr>
<td>PaO₂, torr</td>
<td>347 ± 80</td>
<td>320 ± 70</td>
<td>373 ± 62</td>
</tr>
<tr>
<td>PaCO₂, torr</td>
<td>37 ± 4</td>
<td>38 ± 3</td>
<td>39 ± 5</td>
</tr>
<tr>
<td>pH</td>
<td>7.42 ± 0.03</td>
<td>7.33 ± 0.02†</td>
<td>7.41 ± 0.04</td>
</tr>
<tr>
<td>Base, mmol/l</td>
<td>−0.5 ± 0.5</td>
<td>−5.6 ± 0.9†</td>
<td>−0.3 ± 0.7</td>
</tr>
<tr>
<td>HCO₃⁻, mmol/l</td>
<td>23 ± 1</td>
<td>19 ± 1†</td>
<td>23 ± 1</td>
</tr>
<tr>
<td>Hemoglobin, g/dl</td>
<td>14.6 ± 0.5</td>
<td>12.6 ± 0.6†</td>
<td>14.7 ± 0.4</td>
</tr>
<tr>
<td>SaO₂, %</td>
<td>97 ± 1</td>
<td>95 ± 3</td>
<td>97 ± 2</td>
</tr>
<tr>
<td>COHb, %</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

*Significant difference from Sham-CTL at the same time-point (ANOVA; post hoc Tukey-Kramer HSD test; $P < 0.05$). †Significant difference from baseline values (repeated-measures MANOVA; $P < 0.001$). ‡Significant difference from Sham-CTL and CPB-Air groups at the same time-point (ANOVA; post hoc Tukey-Kramer HSD test; $P < 0.05$). CBP, cardiopulmonary bypass; COHb, carboxyhemoglobin; SaO₂, hemoglobin saturation.
detectable in the CPB-CO group (peak ~1%). Using repeated-measures MANOVA, we detected a significant experimental group and time effect on diastolic arterial pressure and hemoglobin levels (P < 0.001). In the CPB-Air and CPB-CO groups, the lowest Hb value was observed during CPB (~7.6 g/dl), with no difference between these two groups. The Sham-CTL group showed a progressive lowering of hemoglobin levels, likely as a result of hemodilution. Over time, all experimental groups developed slight metabolic acidosis reflected by reductions in bicarbonate and base levels. There were no significant differences in hemodynamic variables or blood-gas analyses between the CPB-Air and CPB-CO groups during recovery following CPB (Table 1). Hemodynamic management was not different between the two CPB groups during the 1-h bypass run, at the time of weaning from CPB, or during the 90-min recovery period. Accordingly, there were no differences in the use of inotropic or vasopressor agents, or volume infusion between experimental groups.

**DIAm contractility and susceptibility to fatigue after CPB.** DIAm contractile (P<, P<o) or fatigue (MFI) properties in the Sham-CTL rats were consistent with previous studies in rats (25, 27), indicating that the short exposure to mechanical ventilation had minimal effects on DIAm contractile or fatigue properties. Both P< and P<o were significantly lower in the CPB-Air group compared with Sham-CTL rats. Overall, specific force declined ~25% after CPB (Fig. 1), thus, the ratio of twitch to tetanic force was not different across groups (51 ±2%).

Diaphragm muscle fatigability to 40-Hz repetitive stimulation was independently assessed in separate muscle segments. Isometric force generated after 2 min of repetitive stimulation was ~30% of initial in Sham-CTL rats (Table 2). Although the relative decline in muscle force during repetitive supramaximal stimulation (2 min) in the CPB-Air group was greater than in the Sham-CTL, this difference did not reach statistical significance (P = 0.12).

![Fig. 1. Isometric contractile properties of the diaphragm muscle of sham-operated (Sham-CTL) rats that underwent mechanical ventilation and vascular cannulation without cardiopulmonary bypass (CPB) compared with two experimental groups that underwent normothermic CPB for 1 h followed by a 90-min recovery period: one group was exposed to air (CPB-air group) during CPB, and the other group was exposed to low-dose inhaled CO (250 ppm in air; CPB-CO group). Specific force was determined by normalizing maximal force to muscle cross-sectional area (see MATERIALS AND METHODS). In the CPB-Air group, a significant decrease occurred in isometric twitch (P<) and maximum tetanic force (P<o). Application of CO restored diaphragm muscle contractile properties. *Significant difference from Sham-CTL group. ‡Significant difference from CPB-Air group. All comparisons were performed using ANOVA, followed by post hoc Tukey-Kramer honestly significant difference test (HSD) (P < 0.05).

**Table 2. Fatigue properties of the diaphragm muscle following CPB**

<table>
<thead>
<tr>
<th></th>
<th>Sham-CTL (n = 6)</th>
<th>CPB-Air (n = 6)</th>
<th>CPB-CO (n = 6)</th>
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<tbody>
<tr>
<td>Fatigue index, %</td>
<td>27 ± 2</td>
<td>22 ± 4</td>
<td>29 ± 2‡</td>
</tr>
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</table>

‡Significant difference from CPB-Air group (ANOVA, post hoc Tukey-Kramer HSD test; P < 0.05).

**Neuromuscular transmission failure in the DIAm after CPB.** Initial muscle force elicited by nerve stimulation in Sham-CTL rats was comparable to previous results using control rats (25, 27). After 2 min of repetitive activation, the forces elicited by nerve stimulation became ~30% lower than those elicited by direct muscle stimulation in Sham-CTL rats (Fig. 2A). These results are also similar to previous studies in control rats (25, 27), indicating that mechanical ventilation did not seem to affect neuromuscular transmission in the rat DIAm. During 2 min of repetitive stimulation, there was a noticeably larger difference (approximately twofold) between forces generated by indirect (nerve) and direct muscle stimulation in CPB-Air rats compared with the difference between these forces in the Sham-CTL group (Fig. 2B). Accordingly, the relative contribution of NMTF to DIAm fatigue was significantly higher in the CPB-Air (>75% at 2 min) compared with Sham-CTL group (~60%, Fig. 3). Using repeated-measures MANOVA, we found that there was a significant group vs. time interaction, such that after 45 s of repetitive stimulation, neuromuscular transmission was significantly impaired in the CPB-Air group compared with Sham-CTL. These results indicate that after 1 h of CPB followed by a 90-min recovery period, neuromuscular transmission in the rat DIAm was significantly impaired.

**Effect of CO on muscle contractility and fatigability after CPB.** Application of low-dose inhaled CO (CPB-CO) restored muscle-specific force to control values (P > 0.05 vs. Sham-CTL; Fig. 1), indicating that low-dose inhaled CO was effective in partially mitigating the acute negative effects of CPB on DIAm contractile properties. Furthermore, CO treatment reduced the relative decline in muscle force generation evoked by 2-min of repetitive stimulation, resulting in a small, but statistically significant difference in DIAm fatigability in the CPB-CO group compared with the CPB-Air group but not the Sham-CTL group (Table 2).

**Effect of CO on neuromuscular transmission failure.** With repetitive stimulation, the difference in forces generated by indirect (nerve) and direct muscle stimulation was significantly reduced in the CPB-CO group compared with CPB-Air group (~40% after 2 min; Fig. 2C). Accordingly, the relative contribution of NMTF to DIAm fatigue was significantly lower in the CPB-CO group (~60% at 2 min) compared with the CPB-Air group (Fig. 3). Using repeated-measures MANOVA, there was no significant difference between the CPB-CO and Sham-CTL groups at any time point. NMTF was significantly different between the CPB-CO and CPB-Air groups after 45 s of repetitive stimulation and for the remainder of the 2-min stimulation period.

**DISCUSSION**

The present study demonstrates that CPB in rats: 1) has significant negative effects on DIAm force generation and neuromuscular transmission; and, 2) CO treatment during CPB
largely mitigates these deleterious effects. The effects of CPB were both rapid in onset and significant. A 1-h exposure to CPB followed by a 90-min recovery period was sufficient to decrease DIAm isometric force ($P_t$, $P_o$) and impair neuromuscular transmission. These deleterious effects of CPB were abated by administration of low-dose inhaled CO during CPB, restoring specific twitch force, muscle fatigue index, and neuromuscular transmission to control levels. Importantly, mechanical ventilation for the same duration of exposure resulted in minimal neuromuscular effects.

Neuromuscular response to CPB. Previous studies suggest that CPB results in many postoperative complications, including pulmonary dysfunction and respiratory failure (18, 31, 33). However, there is a paucity of data concerning the effects of CPB on the neuromuscular system. Our findings that CPB caused a rapid decrease of muscle-specific forces (both $P_t$ and $P_o$) are in agreement with the significant impairment of ventilatory muscle contractile performance reported in septic shock (8, 46). Although DIAm contractile properties were generally consistent with previous studies (25, 27), $P_o$ was slightly lower in the mechanically ventilated rats compared with previous reports in normal control rats without tracheal or vascular instrumentation (16.8 vs. 19.3 N/cm², respectively). These minimal effects may have resulted from the mild levels of metabolic acidosis observed in all groups in this study. Regardless, because of the highly invasive nature of CPB, we believe that the Sham-CTL group is the appropriate comparison group in these studies. Furthermore, there is presently only indirect evidence for impaired neuromuscular transmission following hypothermic CPB (4). Thus, our findings that 1 h of normothermic CPB impairs neuromuscular transmission are novel and likely clinically relevant. Future studies can extend these observations by including a time course for the effects of CPB on the neuromuscular system, as the longer duration of CPB (as may be seen clinically in course of multivessel coronary artery bypass procedures or intracardiac valvular repair) may result in more extensive effects.

The impaired force generation and neuromuscular transmission following CPB may reflect specific cellular, metabolic, immune, and hemodynamic defects, which interfere with processes necessary for normal force generation (19).
quence of CPB-evoked events includes excessive thrombin formation; production of anaphylotoxins C3a and C5a with anaphylactic and chemotactic activity; production of endotoxins and proinflammatory cytokines, such as TNF-α and the interleukins IL-1β and IL-6 (3, 36); and excessive release of nitric oxide (NO) through the inducible form of the enzyme NO synthase (33, 45). Any of these factors may be responsible for the worsening contractile and neuromuscular transmission properties of the rat DIAm post-CPB (22, 47). Future studies are needed to elucidate the underlying mechanisms responsible for the neuromuscular effects of CPB.

Systemic effects of CO treatment. Exogenous application of CO can exert both anti-inflammatory and antiapoptotic effects in cells and tissues (17). We found that low-dose, inhaled CO treatment during CPB was sufficient to restore Pn, MFI, and NMTF to control levels. Although CO treatment partially reversed the effect of CPB on Pn, there was no statistically significant difference between the CPB-CO group compared with CPB-Air or Sham-CTL groups (Fig. 1). In post hoc analyses, we confirmed that our sample size (n = 6 per group) provided ⩾90% power to detect a 1.6 SD difference for most measurements, and specifically 72% power in the case of Pn comparisons. Future studies can extend these observations by including different durations of CO exposure and starting treatment after the onset of CPB-induced neuromuscular effects.

Exogenously applied CO mitigates inflammatory effects in models of acute and chronic inflammation (28, 29, 48). It is widely accepted that CO, an endogenous by-product of heme metabolism, is produced in injured tissue via induction of heme-oxygenase-1 activity, but it is not known if induction of heme-oxygenase-1 results in correspondingly increased CO levels in tissues, such as the DIAm post-CPB. Generally, a physiologically relevant concentration of CO in body fluids is in the range of 100–500 ppm (48). At these concentrations, CO inhibited the production and secretion of cytokines, such as TNF-α, IL-1, and IL-2 (proinflammatory), and stimulated the synthesis of the anti-inflammatory cytokine IL-10 (32). In both rodents and swine, inhalation of a very low concentration of CO (75 ppm) before abdominal surgery attenuated postoperative ileus and hastened postoperative recovery (29). In addition, brief CO inhalation at similarly low concentrations during mechanical ventilation reduced lung injury caused by combination of high tidal volume ventilation and bacterial lipopolysaccharide treatment in rat and mouse models (11, 18). Accordingly, in our experiments, brief (1 h) inhalation of CO at low concentrations (250 ppm, or 8.93 μM/g mixture) during CPB prevented the negative effects of CPB on the neuromuscular system. Several other pharmacological strategies have been used to mitigate the cytotoxic consequences of CPB, including anti-inflammatory glucocorticoids, heparin, and other glycosaminoglycans that prevent blood clotting, antioxidants that reduce oxygen free radicals produced by endogenous cytokines (33), and administration of NO donors such as sodium nitroprusside (20). Further studies are necessary to elucidate whether the effects of CO treatment on the neuromuscular system result from anti-inflammatory or other systemic neurohumoral activity.

Several lines of evidence suggest that CO is an important chemical signal that modulates neurotransmission (24), consistent with our findings at the DIAm neuromuscular junction (Fig. 3). For instance, CO regulates expression of the neuronal α3 isof orm of Na+-K+-ATPase in rat cerebellar slices (30, 41), participates in induction of long-term potentiation in rat superior cervical ganglion (2) and mediates tonic retrograde up-regulation of neurotransmitter release at the frog neuromuscular junction (43). CO also controls the level of resting membrane potential within the gut smooth muscle (14, 40, 44), and its presence is essential for normal inhibitory neuromuscular transmission in these muscles (16). The exact mechanisms regulating the effect(s) of CO on neuromuscular transmission at the DIAm, however, are presently unknown. For instance, inhibition of CO synthesis by inhibitors of heme oxygenase could be used to evaluate directly whether CO depletion has deleterious effects on the DIAm.

As expected, administration of low-dose inhaled CO resulted in slightly elevated carboxyhemoglobin levels (~1%; Table 1). However, oxygenation was not impaired since arterial gas Po2 and SaO2 were unchanged relative to the Sham-CTL group. In addition, there was no evidence of alterations in acid/base balance, suggestive of tissue hypoxia in the CO treatment group. Thus, low-dose CO treatment may have therapeutic importance in preventing the systemic neurohumoral response associated with CPB.

Perspectives and Significance

Patients undergoing CPB can manifest unilateral or bilateral phrenic nerve paresis, with subsequent DIAm dysfunction and difficulty in weaning from mechanical ventilation (10, 13, 21). Diaphragm dysfunction is often attributed to hypothermic damage to phrenic nerves from topical cooling during surgery (5, 26). However, other factors such as direct surgical trauma, a generalized systemic response following blood contact with nonphysiological surfaces of the extracorporeal circuit, endotoxemia, and nerve and/or muscle ischemia may all contribute to DIAm dysfunction post-CPB. We have provided new information demonstrating that CPB has a significant effect on the neuromuscular system, reducing isometric force generation by muscle fibers, and impairing neuromuscular transmission. In addition, administration of low-dose inhaled CO largely ameliorates these negative effects of CPB. Further studies are needed to ascertain the underlying mechanisms of CPB-induced neuromuscular dysfunction and to establish the role of CO therapy in this context.

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GRANTS

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DISCLOSURES

No conflicts of interest are declared by the authors.

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