Sympathetic and cardiovascular responses to venous distension in an occluded limb

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Cui J, Leuenberger UA, Gao Z, Sinoway LI. Sympathetic and cardiovascular responses to venous distension in an occluded limb. Am J Physiol Regul Integr Comp Physiol 301: R1831–R1837, 2011. First published September 21, 2011; doi:10.1152/ajpregu.00170.2011.—We recently showed that a fixed volume (i.e., 40 ml) of saline infused into the venous circulation of an arterially occluded vascular bed increases muscle sympathetic nerve activity (MSNA) and blood pressure. In the present report, we hypothesized that the volume and rate of infusion would influence the magnitude of the sympathetic response. Blood pressure, heart rate, and MSNA were assessed in 13 young healthy subjects during forearm saline infusions (arrested circulation). The effects of different volumes of saline (i.e., 2%, 3%, 4%, or 5% forearm volume at 30 ml/min) and different rates of infusion (i.e., 5% forearm volume at 10, 20, or 30 ml/min) were evaluated. MSNA and blood pressure responses were linked with the infusion volume. Infusion of 5% of forearm volume evoked greater MSNA responses than did infusion of 2% of forearm volume (Δ11.6 ± 1.9 vs. Δ3.1 ± 1.8 bursts/min and Δ332 ± 105 vs. Δ38 ± 32 units/min, all P < 0.05). Moreover, greater MSNA responses were evoked by saline infusion at 30 ml/min than 10 ml/min (P < 0.05). Sonographic measurements confirmed that the saline infusions induced forearm venous distension. The results suggest that volume and rate of saline infusion are important factors in evoking sympathetic activation. We postulate that venous distension contributes to cardiovascular autonomic adjustment in humans.

When humans stand up, blood is displaced into the capacitance vessels in the dependent regions of the body (13, 26); despite this translocation, blood pressure is maintained (27). It is widely accepted that cardiopulmonary (27) and arterial (29) baroreceptor disengagement occurs with standing and leads to tachycardia and peripheral vasoconstriction. In the process, orthostatic hypotension is prevented. However, a necessary stimulus for baroreceptor disengagement is a fall in blood pressure. It is thus somewhat surprising that mean arterial blood pressure (MAP) frequently does not fall with standing and may even increase slightly (12). In this report, we examine whether local peripheral venous distension is capable of raising blood pressure. We speculate that venous distension can raise blood pressure, even in the absence of arterial or cardiopulmonary baroreceptor disengagement. If so, this would suggest that this system helps maintain hemostasis during orthostatic stress. The concept that peripheral veins can serve as autonomic sensor organs is not new. Clearly, lower limbs and abdominal veins become distended with orthostatic challenge (13). Meanwhile, animal experiments suggest that distension of femoral-saphenous veins (10, 24), large abdominal veins (2), and the veins in the triceps surae muscle (15) increases group III and IV afferent discharge and leads to enhanced sympathetic efferent discharge (2, 11). Recently, we reported that infusion of 40 ml of saline into the veins of the occluded human forearm evokes significant systemic sympathetic activation [i.e., muscle sympathetic nerve activity (MSNA)] and a blood pressure rise (7). In this experimental model, peripheral venous distension should be induced without changes in central blood volume. We postulate that venous distension stimulates afferents in or close to the vein wall and, in turn, evokes sympathetic activation. However, in our earlier report (7), we did not document venous distension. Moreover, we did not determine the physiological characteristics of this reflex. Specifically, we did not explore the relationship between infusate volume and rate and the magnitude of reflex responses (e.g., MSNA and blood pressure). As with other reflexes [e.g., baroreflex (21)], determination of these factors is crucial in understanding the role this system plays in autonomic regulation.

Prior animal studies suggest that the afferents in close proximity to femoral-saphenous veins in cats respond to the magnitude and rate of change in venous pressure (10). In the present study, we test the hypothesis that, in human subjects, the volume of the infusate and the rate of infusion would contribute to the magnitude of the autonomic reflex response.

Methods

Subjects

Thirteen subjects [7 men and 6 women, 26 ± 1 (SE) yr] participated in the study. All were of normal height (172 ± 2 cm) and weight (73 ± 3 kg), normotensive (supine blood pressures <140/90 mmHg), and in good health, and none were taking medication. Subjects refrained from caffeine, alcohol, and exercise 24 h prior to the study. The experimental protocol was approved by the Institutional Review Board of the Milton S. Hershey Medical Center and conformed with the Declaration of Helsinki. The purposes and risks of the protocol were explained to each subject before written informed consent was obtained.

Measurements

Forearm volume (i.e., from elbow to wrist) was assessed by water displacement. Beat-by-beat blood pressure was recorded from a finger (Finometer, Finapres Medical Systems, Amsterdam, The Netherlands), with resting values verified by auscultation of the brachial artery (SureSigns VS3, Philips, Philips Medical System) from the nontreated arm. Heart rate was monitored from the electrocardiogram (Cardicap 5, Datex-Ohmeda, GE Healthcare). Respiratory frequency was monitored using piezoelectric pneumography, and multifiber recordings of MSNA were obtained with a tungsten microelectrode inserted into the peroneal nerve of a leg. A reference electrode was placed subcutaneously 2–3 cm from the recording electrode. The
Table 1. Baseline cardiovascular variables and MSNA in trials with different infusion volumes

<table>
<thead>
<tr>
<th>Infusion Volume</th>
<th>2%</th>
<th>3%</th>
<th>4%</th>
<th>5%</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP, mmHg</td>
<td>127 ± 4</td>
<td>128 ± 4</td>
<td>130 ± 4</td>
<td>128 ± 4</td>
<td>0.72</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>61 ± 3</td>
<td>62 ± 2</td>
<td>64 ± 2</td>
<td>63 ± 2</td>
<td>0.11</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>84 ± 3</td>
<td>84 ± 2</td>
<td>86 ± 2</td>
<td>84 ± 2</td>
<td>0.09</td>
</tr>
<tr>
<td>Heart rate, bpm/min</td>
<td>68 ± 2</td>
<td>66 ± 3</td>
<td>66 ± 3</td>
<td>66 ± 3</td>
<td>0.27</td>
</tr>
<tr>
<td>MSNA bursts/min</td>
<td>19.2 ± 1.9</td>
<td>20.3 ± 1.6</td>
<td>18.1 ± 1.8</td>
<td>21.1 ± 2.1</td>
<td>0.41</td>
</tr>
<tr>
<td>units/min</td>
<td>292 ± 42</td>
<td>333 ± 56</td>
<td>284 ± 46</td>
<td>345 ± 65</td>
<td>0.08</td>
</tr>
<tr>
<td>Respiration cycles, min⁻¹</td>
<td>16.9 ± 1.3</td>
<td>16.5 ± 1.3</td>
<td>17.4 ± 1.0</td>
<td>17.1 ± 1.2</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Values are means ± SE of 12 subjects. SBP, DBP, and MAP, systolic, diastolic, and mean arterial blood pressures (measured by automated sphygmomanometer from an upper arm); MSNA, muscle sympathetic nerve activity. P values were determined by 1-way repeated-measures ANOVA. There is no significant difference in measurements among trials.
with the burst sound from the audio amplifier. These bursts were further evaluated by a computer program that identified bursts on the basis of fixed criteria, including an appropriate latency following the R wave of the electrocardiogram (6). Integrated MSNA was normalized by assigning a value of 100 to the mean amplitude of the top 10% largest bursts during the 6-min baseline period. The MSNA signal was normalized to reduce variability between subjects attributed to factors including needle placement and signal amplification. Total MSNA was identified from burst area of the integrated neurogram. MAP was calculated from the Finometer waveform during each trial, while the baseline MAP was verified by an automated sphygmomanometer from an upper arm.

The mean values of MSNA, MAP, and heart rate over the 6-min freely perfused baseline, W-E occlusion, the last 3 min of the preinfusion, the last 30 s during the infusion, the first and the second 30 s of postinfusion, and the 2nd and 3rd min of postinfusion were analyzed. Because the peak MSNA and blood pressure responses occurred toward the end of the infusion period in all trials, the mean values obtained during the last 30 s of infusion and the first 30 s of the postinfusion period (total 1 min; “Max” in Fig. 1) were considered as the maximal responses. The changes (Δ) from the preinfusion to the Max response were also used for the comparison.

Statistics

Differences in the absolute values of cardiovascular variables among the baselines of the trials in each visit were evaluated via repeated-measures one-way ANOVA. The study for the effects of the infusion volume had two within-subject factors: interventions in the protocol (factor 1, e.g., infusion) and volume (factor 2, i.e., 4 levels: 2%, 3%, 4%, and 5%). The absolute values of cardiovascular variables and MSNA were used to examine the effects of the two factors via repeated-measures two-way ANOVA. When appropriate, Tukey’s post hoc analyses were employed. To further examine the effects of volume on responses to the infusion between the trials, the differences among the changes (Δ) from the preinfusion to the Max response were evaluated via repeated-measures one-way ANOVA. Moreover, the relationships between the infusion volume and MSNA and MAP responses to the infusion (i.e., the changes) were analyzed via linear regression analysis. In a similar manner, the cardiovascular variables and MSNA in the infusion rate study were compared. Values are means ± SE. P < 0.05 was considered statistically significant.

RESULTS

Effects of Infusion Volume

The measured forearm volume of the 12 subjects was 1,027 ± 23 ml (range 717–1,447 ml). The actual volumes of infused saline in the 2%, 3%, 4%, and 5% forearm volume trials were 20.5 ± 1.3, 30.5 ± 1.9, 40.8 ± 2.5, and 49.8 ± 2.5 ml, respectively. Baseline MSNA and cardiovascular variables were similar during the four trials (Table 1). Representative recordings of heart rate, integrated MSNA, and blood pressure during saline infusion of 5% of forearm volume (30 ml/min) are shown in Fig. 1. The absolute MSNA and the cardiovascular variables for the four trials are shown in Fig. 2. The W-E occlusion procedure and preinfusion ischemia did not induce significant changes in the variables in any of the trials. The 5% and 4% infusion with different volumes

<table>
<thead>
<tr>
<th>Infusion Volume</th>
<th>2%</th>
<th>3%</th>
<th>4%</th>
<th>5%</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔMAP, mmHg</td>
<td>1.8 ± 0.8</td>
<td>3.9 ± 1.1</td>
<td>6.8 ± 1.4*</td>
<td>9.9 ± 2.0*</td>
<td>0.004</td>
</tr>
<tr>
<td>ΔHeart rate, beats/min</td>
<td>1.0 ± 0.4</td>
<td>1.0 ± 1.0</td>
<td>1.1 ± 1.0</td>
<td>3.7 ± 1.5</td>
<td>0.124</td>
</tr>
<tr>
<td>ΔMSNA, bursts/min</td>
<td>3.1 ± 1.8</td>
<td>4.7 ± 1.6</td>
<td>11.0 ± 1.9*</td>
<td>11.6 ± 1.9†</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>units/min</td>
<td>38 ± 32</td>
<td>72 ± 36</td>
<td>192 ± 33</td>
<td>332 ± 105†</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Values (means ± SE) are changes from preinfusion to Max period (see Fig. 1). MAP was measured from a finger with a Finometer device. P values were determined by 1-way repeated-measures ANOVA. *P < 0.05 vs. 2%. †P < 0.05 vs. 0.05 vs. 3%.
forearm volume trials led to increases in MAP and MSNA. Saline infusions of 2% and 3% of forearm volume did not induce significant increases in MSNA. Heart rate did not change significantly in any of the four trials.

The infusion of a larger volume (e.g., 5%) of saline induced greater MSNA and MAP increases (i.e., change from preinfusion to Max period) than the infusion of smaller volumes (e.g., 2%; Table 2). Moreover, relationships were identified between the volume infused and the MAP increase (R = 0.55, P < 0.001) and between the volume infused and the MSNA increase (burst rate: R = 0.51, P < 0.001; total activity: R = 0.50, P < 0.001). No relationship was noted between the volume infused and the heart rate response (R = 0.25, P = 0.08).

Effects of Infusion Rate

The baseline cardiovascular variables and MSNA were similar before the three rate trials (Table 3). The cardiovascular variables and MSNA responses during the three trials are shown in Fig. 3. MSNA and MAP increased significantly toward the end of the infusions in all the rate trials. In the 20 ml/min trial, heart rate increased significantly toward the end of infusion. The MSNA (expressed as burst rate) response was greater during the 30 ml/min than the 10 ml/min infusion (Table 4). There was no significant difference in MAP and heart rate responses between the trials. No relationships were noted between infusion rate and MAP increase (ΔMAP; R = 0.34, P = 0.06; total activity: R = 0.30, P = 0.11), or between infusion rate and heart rate response (ΔHR; R = −0.20, P = 0.29).

Forearm venous diameters were successfully obtained in six subjects during the rate studies. One example of changes in antecubital forearm venous diameter during saline infusion is shown in Fig. 4. At the end of the infusion, the average increase in venous diameters of the three trials in response to the saline infusion was 1.4 ± 0.2 mm. Diameter increased more in response to infusion at 30 ml/min than 10 ml/min (Table 5).

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Table 3. Baseline cardiovascular variables and MSNA in trials with different infusion rates

<table>
<thead>
<tr>
<th>Infusion Rate</th>
<th>10 ml/min</th>
<th>20 ml/min</th>
<th>30 ml/min</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP, mmHg</td>
<td>123 ± 3</td>
<td>121 ± 3</td>
<td>123 ± 3</td>
<td>0.68</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>63 ± 2</td>
<td>64 ± 2</td>
<td>63 ± 3</td>
<td>0.74</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>83 ± 2</td>
<td>83 ± 2</td>
<td>83 ± 2</td>
<td>0.97</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>72 ± 3</td>
<td>72 ± 2</td>
<td>71 ± 2</td>
<td>0.35</td>
</tr>
<tr>
<td>MSNA bursts/min</td>
<td>15.7 ± 3.1</td>
<td>19.1 ± 3.4</td>
<td>17.1 ± 3.0</td>
<td>0.08</td>
</tr>
<tr>
<td>units/min</td>
<td>50 ± 30</td>
<td>379 ± 57</td>
<td>324 ± 56</td>
<td>0.07</td>
</tr>
<tr>
<td>Respiration cycles, min⁻¹</td>
<td>17.2 ± 1.2</td>
<td>17.1 ± 1.0</td>
<td>16.8 ± 1.3</td>
<td>0.81</td>
</tr>
</tbody>
</table>

Values are means ± SE of 10 subjects. Blood pressures were measured by an automated sphygmomanometer from an upper arm. P value was determined by 1-way repeated-measures ANOVA. There is no significant difference in measurements among trials.

Table 4. Cardiovascular and MSNA responses to saline infusion with different rates

<table>
<thead>
<tr>
<th>Infusion Rate</th>
<th>10 ml/min</th>
<th>20 ml/min</th>
<th>30 ml/min</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔMAP, mmHg</td>
<td>8.0 ± 1.4</td>
<td>13.3 ± 2.9</td>
<td>10.0 ± 2.5</td>
<td>0.183</td>
</tr>
<tr>
<td>ΔHeart rate, beats/min</td>
<td>5.4 ± 3.3</td>
<td>6.2 ± 2.8</td>
<td>1.9 ± 1.8</td>
<td>0.046</td>
</tr>
<tr>
<td>ΔMSNA bursts/min</td>
<td>4.9 ± 2.5</td>
<td>9.0 ± 1.7</td>
<td>10.6 ± 2.0*</td>
<td>0.049</td>
</tr>
<tr>
<td>units/min</td>
<td>116 ± 55</td>
<td>253 ± 54</td>
<td>264 ± 78</td>
<td>0.075</td>
</tr>
</tbody>
</table>

Values (means ± SE) are changes from preinfusion to Max period (see Fig. 1). MAP was measured from a finger with a Finometer device. P values were determined by 1-way repeated-measures ANOVA. *P < 0.05 vs. 10 ml/min.
MSNA and blood pressure responses was linked to the infused volume. Moreover, the sonographic measurements verified that the infused saline did, in fact, induce forearm venous distension. The relative increases in diameter and area were ~40% and ~90%, respectively. These anatomic magnitudes of distension fit nicely with the volume of blood translocation to the lower limbs with head-up tilt (3). This suggests that the magnitude of distension shown in this report may be sufficient to evoke autonomic adjustment in humans with standing.

The effects of varying the infusion rate suggest for the first time in humans that rapid venous filling can evoke this reflex. These findings are consistent with prior animal studies (10) and suggest that the rate of mechanical venous distension influences the rate of autonomic adjustments. Our results also suggest that the responses are not due to an increase in interstitial fluid alone. If the observed responses were caused by an increase in interstitial fluid alone, then the infusion rate should not affect the magnitude of the responses at the end of the infusion. Moreover, because saline diffuses into the interstitial space during and after the infusion period, the increase in interstitial fluid should be greater during the postinfusion period than at the end of infusion. However, MSNA and MAP responses decreased during the postinfusion period. Unfortunately, the present data do not afford the opportunity to examine whether interstitial fluid plays a role in modifying the venous distension reflex. This issue will be addressed in future studies.

Although heart rate did significantly increase toward the end of infusion in one trial (Fig. 3), it did not significantly increase in other trials in the present study. This was different from our previous findings (7). Multiple trials in a random order were performed in each of the two visits of the present study, while only one trial was performed in each visit in our previous study. Thus some factors, such as adaptation, might cause the difference in the heart rate response to the infusion.

**Perspectives and Significance**

What is the physiological significance of these results? With orthostatic challenge (e.g., head-up tilt), sympathetic outflow increases and peripheral vasoconstriction are noted. These changes occur without a drop in MAP (12). We suspect that the small changes in baroreceptor stimulation during upright posture do not entirely explain the increases in sympathetic outflow and peripheral vasoconstriction. When humans suddenly stand, the venous valves in the lower limb remain closed until a certain level of distension occurs. Under normal arterial flow and perfusion, the triceps surae muscle evoke an increase in thin fiber muscle afferent discharges. Data from our prior report in humans (7) suggest that venous volume infusion into an occluded forearm is a sufficient stimulus to evoke reflex increases in MSNA and blood pressure. The present report extends these findings by suggesting that the rate and the magnitude of the distension are important determinants of this peripheral autonomic reflex response.

The following observations regarding the pattern of MSNA activation should be noted: 1) the periods of ischemia with 2%, 3%, 4%, and 5% trials were not dramatically different (i.e., ~10.6–11.6 min), whereas MSNA responses were different for different volumes, and 2) in trials associated with a rise in MSNA and blood pressure, the largest effects were noted during the midportion of the period of ischemia (Fig. 1). These observations suggest that the autonomic responses were not related to the period of ischemia and/or the mental stress associated with the paradigm.

The present data clearly show for the first time in humans that this reflex has a threshold and that reflex stimulation evokes graded adjustments. Specifically, the magnitude of MSNA and blood pressure responses was linked to the infused volume. Moreover, the magnitude of the MSNA and blood pressure responses was linked to the infused volume; and 3) the infusion rate also had an effect on the size of the vein and the magnitude of the reflex response. These observations support the hypothesis that limb venous distension evokes sympathetic activation in humans in a predictable and hemodynamically logical fashion.

Prior studies in animal models suggest that abdominal veins (2), as well as large (10, 24, 30, 34) and small (1, 28, 32, 33) limb veins, are innervated by thinly myelinated and unmyelinated afferent nerves. Afferent fibers such as these are noted to respond to chemical and mechanical stimuli (16–19, 22, 23). Important physiological experiments by Haouzi et al. (14, 15) suggest that vasodilating substances infused into the arterial supply of the triceps surae muscle evoke an increase in thin fiber muscle afferent discharges. Data from our prior report in humans (7) suggest that venous volume infusion into an occluded forearm is a sufficient stimulus to evoke reflex increases in MSNA and blood pressure. The present report extends these findings by suggesting that the rate and the magnitude of the distension are important determinants of this peripheral autonomic reflex response.

**DISCUSSION**

The main findings of this study are as follows: 1) saline infusion of 5% forearm volume into an occluded arm induced forearm vein distension; 2) the magnitudes of the increase in MSNA and MAP were linked to the infused volume; and 3) the infusion rate also had an effect on the size of the vein and the magnitude of the reflex response. These observations support the hypothesis that limb venous distension evokes sympathetic activation in humans in a predictable and hemodynamically logical fashion.

Table 5. **Diameter of the vein at the antecubital fossa**

<table>
<thead>
<tr>
<th>Trial</th>
<th>Infusion Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 ml/min</td>
</tr>
<tr>
<td>Freely perfused baseline</td>
<td>4.1 ± 0.4</td>
</tr>
<tr>
<td>Maximal change from baseline</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>Maximal change from preinfusion</td>
<td>1.5 ± 0.4</td>
</tr>
</tbody>
</table>

Values are means ± SE of 6 subjects. Diameter (mm) of the vein was measured during freely perfused baseline, preinfusion period, and Max period. Saline infusion into occluded arm induced a significant increase in diameter of the vein in all trials. Maximal change from baseline, diameter increase from freely perfused baseline to the end of the infusion; maximal change from preinfusion, diameter increase from preinfusion period to the end of the infusion. P values were determined by 1-way repeated-measures ANOVA. *P = 0.006 vs. 10 ml/min.
conditions, it takes ~2 min for the lower limb veins to fill with ~600 ml of blood (26). We postulate that our retrograde infusion model simulates this gradual process of venous distension and, thus, has implications for our understanding of human autonomic regulation during postural stress. We would further speculate that limb venous distension in and of itself evokes sympathetic activation and vasoconstriction and a rise in blood pressure. The present data clearly show that the magnitude of MSNA and blood pressure responses was linked to the vascular volume changes in the limbs. Briefly, this reflex is ideally suited to respond to the sustained challenges imposed by upright posture.

It is also interesting to note that whole body heat stress raises MSNA in the absence of a reduction in arterial blood pressure (5, 8, 9). This rise in MSNA was not altered/inhibited by loading cardiopulmonary baroreceptors [rapid systemic saline infusion (15 ml/kg)] (5). Thus the cause of the rise in MSNA is unclear. Non-baroreceptor-related mechanisms (e.g., central activation of the sympathetic nervous system) have been postulated (5). We would suggest that heat-induced peripheral venous vasodilation may in and of itself contribute to the MSNA response.

Study Limitations
In the present study, only four relative volumes with a fixed rate and three rates with a fixed volume were examined. Thus the entire curve of the relationship(s) between either volume or rate and responses (blood pressure or MSNA) could not be determined. The 5% forearm volume infusion (30 ml/min) was estimated from clinical work (20) as well as our previous studies (7). On the basis of our assessment of human subject safety, larger volumes and faster infusion rates were not evaluated.

Based on our previous report (7) and the present data, we speculate that venous distension evokes the sympathetic activation. We propose that arm and leg venous systems behave similarly. However, the afferents in the forearm and leg may have different sensitivities to a variety of stimuli. For example, it is clear that greater cardiovascular responses are evoked by human exercise with upper than lower limb muscles (4, 25). Thus further studies are necessary to examine and compare MSNA and cardiovascular responses to upper and lower limb venous distension.

In conclusion, the present study shows that saline infusion into an occluded arm induces venous distension, evokes sympathetic activation, and raises systemic blood pressure increase. The results suggest that volume and the rate of the saline infusion are important factors in evoking this reflex. These observations support the hypothesis that venous distension in limbs contributes to cardiovascular adjustment in humans.

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GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
J.C. and L.I.S. are responsible for conception and design of the research; J.C., U.A.L., and Z.G. performed the experiments; J.C. and Z.G. analyzed the data; J.C., U.A.L., and L.I.S. interpreted the results of the experiments; J.C. prepared the figures; J.C. and L.I.S. drafted the manuscript; J.C. and L.I.S. edited and revised the manuscript; J.C., U.A.L., Z.G., and L.I.S. approved the final version of the manuscript.

REFERENCES