Leonurine Protects Middle Cerebral Artery Occluded Rats Through Antioxidant Effect and Regulation of Mitochondrial Function

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Background and Purpose—Oxidative stress is known to be involved in ischemic stroke. Intense interest is drawn to the therapeutic potential of Chinese herbs on ischemic stroke because many of them contain antioxidant properties. Leonurine, 1 of the active compounds from purified Herba Leonuri, was studied to evaluate its possible therapeutic effects on ischemic stroke.

Method—Middle cerebral artery occlusion was selected as our model of study. The animals were pretreated with Leonurine orally for 7 days and the surgery was done. One day after surgery, 2,3,5-triphenyltetrazolium chloride staining and neurological deficit score were carried out to evaluate the functional outcome of animals, whereas levels of superoxide dismutase, glutathione peroxidase, and malondialdehyde were analyzed for oxidative stress analysis. For mitochondrial studies, 3 hours after surgery, mitochondria were isolated for analysis of reactive oxygen species production, adenosine triphosphate biosynthesis, oxygen consumption, and respiratory control ratio value.

Result—In in vivo experiments, Leonurine pretreatment reduced infarct volume, improved neurological deficit in stroke groups, increased activities of antioxidant enzymes superoxide dismutase and glutathione peroxidase, and decreased levels from the lipid peroxidation marker malondialdehyde. In terms of mitochondrial modulation, Leonurine inhibited mitochondrial reactive oxygen species production and adenosine triphosphate biosynthesis. Animal studies also demonstrated that the mitochondrial function and redox state were restored by Leonurine treatment.

Conclusions—Leonurine has neuroprotective effects and carries a therapeutic potential of stroke prevention. (Stroke. 2010;41:2661-2668.)

Key Words: antioxidant ■ ischemia ■ Leonurine ■ MCAO

Ischemic stroke is caused by a reduction or complete blockade of blood flow, resulting in the deficiency of glucose and oxygen supply to the territory of the affected region.1 Although thrombolysis is the only currently effective available stroke therapy, it is limited to a small proportion of patients with stroke because it carries the risk of intracranial hemorrhagic transformation.

Increased levels of reactive oxygen species (ROS) are the major cause of tissue injury after cerebral ischemia, in which inactivation of antioxidant enzymes and consumption of antioxidants such that endogenous antioxidant defense mechanisms fail to protect neurons from oxidative damage.2 Oxidative stress is the state of imbalance between the level of antioxidant defense mechanism and production of the free radicals that favor the latter leading to potential damage.3 Brain tissue is particularly susceptible to oxidative damage.4 Therefore, it is believed that pharmacological modification of oxidative damage is one of the most promising avenues for stroke therapy.

The principle role of mitochondria is production of 70% to 80% of high-energy phosphate bond adenosine triphosphate (ATP) for cellular function, so-called the “powerhouse of the cell.” Mitochondria energy metabolism is extremely sensitive to impairment by free radicals and that mitochondrial oxidative stress limits metabolic recovery.3 Damage of mitochondria leads to cell death, because mitochondria are involved in energy metabolism and calcium homeostasis as well as the ability of mitochondria to release apoptotic-promoting proteins such as cytochrome C and apoptosis-inducing factor to initiate the intrinsic pathway of apoptosis.5 Ample evidences have suggested that mitochondria have a central role in neurodegenerative disease.

Recent attention has focused on a series of natural products shown to enhance the survival of neurons and to prevent
Leonurine

Chemical structure of Leonurine.

Leonurine (4-guanidino-n-butyl-syringate; Figure 1) is an alkaloid present in Herba Leonuri. It was reported to have antioxidant properties by its strong superoxide-scavenging ability measured by an electron spin resonance spin trapping technique in vivo. Recently, raw Herba Leonuri was scientifically purified and contains mainly stachydrine (C$_{6}$H$_{10}$O$_{5}$), quercetin (C$_{15}$H$_{11}$O$_{7}$), Kaempferol (C$_{15}$H$_{10}$O$_{6}$), Leonurine (C$_{14}$H$_{21}$N$_{3}$O$_{5}$), and apiagenin (C$_{15}$H$_{13}$O$_{5}$). Our previous study showed that purified Herba Leonuri preserves antioxidant properties and does have a cardioprotective effect on ischemic myocardium through an antioxidant effect. We have also demonstrated that the extract has neuroprotective effects on middle cerebral artery-occluded rats through its antioxidant effect.

Leonurine (Leo15, Leo30, Leo60). The drugs were administered orally once daily. After 1 week of presurgery treatment, stroke was induced in the rats by MCAO, which was previously described. Rats were anesthetized with a ketamine/xylazine mixture (0.1 mL/100 g intraperitoneally); a 2-cm skin incision was made in the midpoint between the left orbit and the external auditory canal. A small burr hole was made with a high-speed microdrill through the outer surface of the skull and after removing the dura, the left middle cerebral artery was exposed. Then, the middle cerebral artery was occluded by electrocauterization from the point where it crossed the inferior cerebral vein to a point proximal to the origin of the lenticulostriate branches. The branches of the middle cerebral artery between these 2 points were occluded as well to ensure complete and permanent occlusion. The wound was closed with sutures. Rectal temperature was maintained at 37±0.5°C by means of a heating blanket throughout the surgery as reported previously. Sham rats underwent a similar procedure with the omission of MCAO. Rats were euthanized 24 hours after surgery for sample collection.

Infarct Volume Measurement

Twenty-four hours after MCAO, 10 rats of each group were euthanized. After the brains had been collected, cerebellum and overlying membranes were removed. The brains were sliced into 8 coronal sections with 2-mm thickness each and stained with a 0.1% solution of 2,3,5-triphenyltetrazolium chloride (TTC) in saline at 37°C for 30 minutes and photographed. The infarct size was measured as previously described.

Evaluation of Neurological Deficit

To evaluate neurological function impairment after stroke insult, a neurological deficit grading system with a scale of zero to 5 was carried out on all of the animals as we described previously.

Superoxide Dismutase Activity Measurement

Cortical tissues from 8 rats of each group were collected 24 hours after MCAO and placed into an ice-cold RIPA lysis buffer (Bioengineering Institute, Nanjing, China) was selected to determine the activity of SOD, glutathione peroxidase (Gpx), and MDA measurement. The Superoxide Dismutase Detection Kit (A001; Nanjing Jichang Bioengineering Institute, Nanjing, China) was selected. The determination of Gpx is based on the manufacturer’s instructions.

GPx Activity Detection

to determine the activity of Gpx from each treatment group, the Glutathione Peroxidase Detection Kit (A005; Nanjing Jichang Bioengineering Institute, Nanjing, China) was selected. The determination of Gpx is based on the manufacturer’s instructions.

Lipid Peroxidation Determination

A Malondialdehyde (MDA) Detection Kit (A003; Nanjing Jichang Bioengineering Institute, Nanjing, China) was selected to determine the MDA level as a marker of lipid peroxidation. Assay was conducted according to the manufacturer’s instructions.

Preparation of Intact Rat Brain Mitochondria

Twenty-four adult male Wistar rats weighing 280 to 300 g were anesthetized with a ketamine/xylazine mixture (0.1 mL/100 g intraperitoneally) and brains were collected. Cortex mitochondria isolation was then carried out as previously described.

Measurement of ROS in Isolated Mitochondria

ROS produced from mitochondria can be probed by 2′,7′-dichlorofluorescin diacetate (DCFDA). A total of 0.1 mg cortical mitochondria from each animal (n=6) was added into 200 μL respiratory buffer consisting of 125 mmol/L KCl, 2 mmol/L...
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substrate 10 mmol/L succinate, complex I inhibitor 5 μmol/L rotenone, 100 mmol/L KCl, 12.5 mmol/L KH2PO4, and 10 mmol/L Tris, pH 7.6. A total of 0.03 mg cortical mitochondria (n=6) was incubated with 1 μL of buffer and 125 μmol/L adenosine diphosphate, complex II substrate 10 μmol/L succinate, complex I inhibitor 5 μmol/L rotenone, and the presence or absence of studied drugs with or without H2O2 under condition of continual shaking for 5 minutes at 24°C. Chemiluminescence emitted by the luciferin–luciferase reaction was then measured using a Varioskan Flash microplate reader.

Mitochondrial Respiration Measurement

Wistar rats (n=3 for each group) were treated with 60 mg/kg per day of Leonurine orally once daily for 7 days. MCAO or sham-operated surgery was done on Day 7. Three hours after the surgery, the animals were euthanized. Left and right cortical mitochondria were isolated and oxygen consumption was measured. Mitochondrial respiration was measured polarographically by monitoring the rate of oxygen consumption in an airtight chamber equipped with a magnetic stirring device using a Clark-type oxygen electrode (Oxygraph System, Hansatech, UK). One milligram of mitochondria was added into the solution as for the H2O2-treated mitochondrial group. A total of 10 mmol/L succinate was added to the solution to energize the mitochondria. The reactions were started by measuring dichlorofluorescein (DCF) fluorescence at an excitation/emission wavelength of 485 nm/526 nm, respectively, for 5 minutes as basal/respiration to the basal rates. Respiratory control ratio (RCR) was then calculated as ratio of mean slopes of states 3 over state 4 respiration slopes.

Statistical Analysis

Data were represented as mean±SEM of at least 3 independent preparations. Statistical analysis was performed by the 1-way analysis of variance with Tukey post hoc test. A difference with P<0.05 was considered statistically significant.

Results

Improved Functional Outcome of Rats Undergoing MCAO by Leonurine

Figure 2A showed the infarct area of each treatment group. As the rats from sham underwent the similar surgical procedure but without the occlusion of middle cerebral artery, there was no infarct area observed in this group of animals. In the animals subjected to ischemic insult by left MCAO, the infarct area was observed in the left cortex and striatum (22.45%±2.88%). Under the treatment of Leonurine (Leo), the infarct volume was reduced in a dose-dependent manner. A total of 30 mg/kg per day of Leonurine treatment could statistically reduce the infarct volume from 22.45%±2.88% to 15.55%±1.58%, whereas 60 mg/kg per day of Leonurine treatment statistically reduced the infarct volume to 13.50%±1.74% (Figure 2B).

In terms of evaluation of neurological function, neurological deficit grading system was carried out for all the animals. The higher the neurological deficit score, the more severe impairment of motor motion. From Table 1, rats from the sham group did not have any neurological deficit, and therefore the animals had a neurological score of zero. For the rats in the vehicle group, they remained with the highest neurological deficit score after the surgery. In agreement with infarct volume measurement, Leonurine treatment could reduce the neurological deficit score in a dose-dependent manner.

Table 1. Neurological Deficit Grading System

<table>
<thead>
<tr>
<th>Groups</th>
<th>Sham</th>
<th>Vehicle</th>
<th>Leo15</th>
<th>Leo30</th>
<th>Leo60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurological deficit score</td>
<td>0</td>
<td>2.50±0.55</td>
<td>2.67±0.52</td>
<td>1.83±0.75*</td>
<td>1.50±0.55*</td>
</tr>
</tbody>
</table>

*P<0.05 Leonurine-treated stroke-operated versus vehicle-treated group.
manner (Table 1), from 2.5 ± 0.55 to 2.67 ± 0.52 (Leo15) and 1.83 ± 0.75 (Leo30, P < 0.05) and 1.50 ± 0.55 (Leo60, P < 0.05). From both infarct volume measurement and neurological deficit grading system, we demonstrated that the dosage of Leonurine at 60 mg/kg per day gave the best therapeutic effects, and therefore we focused on the Leonurine treatment at 60 mg/kg per day for subsequent biochemical and molecular analysis.

**Oxidative Stress Was Ameliorated by Leonurine In Vivo**

Because brain lesion resulted from MCAO occurs at most of the ipsilateral cortex, cortical tissues were collected for molecular analysis. To investigate the changes of endogenous antioxidant system under the influence of Leonurine, activities of cortical SOD and GPx were evaluated (Table 2). The actives of SOD and GPx in healthy rats (without MCAO) were 15.45 ± 5.11 U/mg and 99.20 ± 19.72 U/mg. Stroke insult by MCAO caused a significant reduction in SOD and GPx activities, to 8.31 ± 2.92 U/mg and 49.32 ± 12.10 U/mg, respectively. Leonurine treatment could restore the activities of SOD and GPx to the level comparable to healthy individuals (SOD at 15.41 ± 5.32 U/mg and GPx at 90.11 ± 21.13 U/mg), indicating the protective effect of Leonurine.

The level of MDA is an index of lipid peroxidation; therefore, it is one of the biomarkers for oxidative stress. As shown in Table 2, MDA level increased significantly from 2.61 ± 1.24 nmol/L to 4.92 ± 1.12 nmol/L per milligram of tissue under the stroke insult by MCAO. Leonurine treatment had a significant effect on reducing the oxidative damage level because reduction of the MDA level to 2.59 ± 1.45 nmol/L (per milligram of tissue) in the stroke-operated Leonurine-treated group was observed (Table 2).

**Reduced Mitochondrial ROS Production by Leonurine**

In addition to the total antioxidant system of brain tissue, cortical mitochondria were isolated to elucidate the effect of Leonurine on mitochondrial ROS production.DCFDA is a nonfluorescent ester of the dye fluorescein but in the presence of ROS, DCFDA is oxidized to the fluorescent product. Succinate as mitochondrial electron transport chain (ETC) complex II substrate was used to stimulate mitochondria throughout the study. We demonstrated that Leonurine (10 μmol/L to 1 mmol/L) treatment resulted in a dose-dependent reduction in ROS production over 30 minutes (Figure 3A). Thirty minutes after the reaction, data from each group were collected and analyzed. It showed that after 30 minutes, 10 μmol/L Leonurine treatment could statistically reduce 30% of mitochondrial ROS production (P < 0.05); 46% by 100 μmol/L Leonurine (P < 0.001); 64.31% by 500 μmol/L of Leonurine (P < 0.001); and 75.68% by 1 mmol/L of Leonurine (P < 0.001; Figure 3B). Next, to demonstrate the effect of Leonurine on mitochondrial ROS production under oxidative stress, mitochondria were subjected to 1 mmol/L H2O2 for 5 minutes followed by the treatment of Leonurine and the ROS production was measured. Figures 3C and 3D showed that under the treatment of 1 mmol/L H2O2, total ROS was increased 3-fold significantly (P < 0.001) as compared with the one without H2O2 treatment. Leonurine treatment ameliorated the mitochondrial ROS production in a dose-dependent manner to 64.29% ± 5.24% (P < 0.05) by 10 μmol/L Leonurine, 54.21% ± 6.74% (P < 0.01) by 100 μmol/L Leonurine, 46.28% ± 5.66% (P < 0.001) by 500 μmol/L Leonurine, and 46.24% ± 6.39% (P < 0.001) by 1 μmol/L Leonurine. This indicates that Leonurine has an antioxidant effect under oxidative stress and the effect might have reached a plateau at 500 μmol/L when the mitochondria were subjected to 1 mmol/L H2O2.

**ATP Biosynthesis in Cortical Mitochondria Was Inhibited by Leonurine**

To study the effect of Leonurine on bioenergetics, mitochondrial ATP production was evaluated under the treatment of Leonurine. We demonstrated under physiological condition, Leonurine could have a dose-dependent inhibitory effect on mitochondrial ATP biosynthesis from 10 μmol/L to 1 μmol/L with a significant effect from 500 μmol/L onward. At 500 μmol/L, Leonurine attenuated ATP biosynthesis to 43.59% ± 6.61% (P < 0.01), whereas 1 mmol/L of Leonurine attenuated ATP biosynthesis to 29.10% ± 5.14% (P < 0.001) as seen in Figure 4A. A total of 1 μmol/L H2O2 caused the mitochondrial dysfunction by attenuated the production of ATP to 57.11% ± 6.56% (P < 0.001). A low concentration of Leonurine did not alter the production of ATP under the treatment of H2O2. An observable effect of Leonurine started from 500 μmol/L that it further reduced the ATP production to 36.97% ± 3.03% (P < 0.05) as compared with the succinate-H2O2 treated group. At 1 μmol/L Leonurine, only 25.25% ± 2.01% (P < 0.01) of ATP production was observed (Figure 4B), indicating that Leonurine at high dose might have a metabolic arrest effect to mitochondria.

**Leonurine Improved Mitochondrial Respiratory Rate In Vivo**

Next, we investigated the effects of Leonurine on mitochondrial oxygen consumption (state 3 respiration) and RCR as the parameters of mitochondrial function. Study was carried out in vivo in which the animals were treated with Leonurine and subjected to MCAO. Mitochondria were isolated 3 hours after the MCAO or sham procedure. We demonstrated that a stroke insult by permanent MCAO at the left cortex could drastically reduce left cortical mitochondrial oxygen consumption to 49.26% ± 7.71% (P < 0.05) as seen in Figure 5A. One-week pretreatment of Leonurine at 60 mg/kg per day could enhance cortical mitochondrial oxygen consumption to 144.42% ± 10.37% (P < 0.05) in healthy individuals (Sham + Leo60) and restore the oxygen consumption in the stroke group (stroke + Leo60) to the level
that is almost comparable to the control group (sham; Figure 5A). Likewise, a significant reduction of RCR value of left cortical mitochondria was observed in the vehicle group as compared with the sham group (2.35±0.27 versus 3.61±0.16, P<0.05) as seen in Figure 5B. Leonurine did not alter the RCR value in the sham group but improved the RCR value in the stroke group from 2.35±0.27 to 3.14±0.1 (P<0.05), indicating the effect of Leonurine on preventing mitochondria from being dysfunctional after stroke insult.

Similar to the left cortex, permanent MCAO at the left affected the oxygen consumption from the right cortical mitochondria to 66.15%±17.36% without the changes of

Figure 3. Effect of Leonurine on cortical mitochondrial ROS production determined by changes in DCF oxidation. Kinetic studies on mitochondrial ROS production over 30 minutes were carried out under the treatment of Leonurine in the absence (A) or presence (C) or 1 mmol/L H$_2$O$_2$. B, D, Percentage of mitochondrial ROS production at 30 minutes was analyzed. In the absence and presence of H$_2$O$_2$, Leonurine treatment reduced the ROS production in a dose-dependent manner. Data (n=5) are normalized and expressed as percent respective to the control-Suc (A–B) and control-suc-H$_2$O$_2$ (C–D). *P<0.05; **P<0.01; ***P<0.001 versus control-Suc (A–B) and control-suc-H$_2$O$_2$ (C–D). ###P<0.001 versus control-suc.

Figure 4. Effect of Leonurine on ATP biosynthesis of succinate treated cortical mitochondria in the absence (A) or presence (B) of 1 mmol/L H$_2$O$_2$. A dose-dependent reduction of ATP biosynthesis by Leonurine was observed in both cases. Data (n=6) are normalized and expressed as percent respective to the control (A) and control-suc (B). **P<0.01; ***P<0.001 versus control (A) and control-suc (B). ###P<0.001 versus control-suc.
RCR value. Leonurine treatment improved the right cortical mitochondrial oxygen consumption in both sham and stroke groups to 140.11% and 134.69% (Figure 5C). In contrast, Leonurine does not have any effect on the RCR value of right cortical mitochondria (Figure 5D).

Discussion
The ischemic cascade starts with a drastic disruption of cerebral blood flow that deprives brain cells of oxygen and glucose supply, leading to a reduction in energy production associated with building up toxic metabolites such as glutamate, inflammatory mediators, and free radicals that could ultimately result in neurodegeneration. Several mechanisms postulated for oxidative stress during ischemia, including increased mitochondrial superoxide production by the disruption of ETC, increased arachidonic acid metabolism with free radicals as byproducts, elevated Ca²⁺ by excitotoxicity, and inflammatory response. Oxidative stress does not play in isolation, but taking place in modulating mitochondrial function, necrosis, apoptosis, inflammation, and excitotoxicity. Therefore, drugs that interfere with oxidative stress carry a potential in neuroprotection.

With this rationale, we tested the therapeutic potentials of Leonurine on MCAO-operated rats. We consistently demonstrated left MCAO caused the infarction at the area in left cortical area and corpus striatum shown by TTC staining. Rats with MCAO had severe neurological deficit score throughout the entire study. Treatment of Leonurine at 60 mg/kg per day significantly reduced the infarct volume and alleviated the neurological impairment, indicating the lesser histological damage was observed as compared with the vehicle group.

SOD dismutates superoxide to hydrogen peroxide and oxygen. GPx eliminates hydrogen peroxide, which is potentially converted to other radicals. MDA is a byproduct of oxidation of polyunsaturated fatty acid. It is one of the markers of oxidative stress. The reduced activities of SOD and GPx and enhanced MDA level in the vehicle group imply that severe oxidative stress occurred during permanent MCAO that increased free radical activity and reciprocally reduced endogenous antioxidant occurring during cerebral ischemia. Our results are consistent with previous work. With Leonurine treatment, enhanced activities of SOD and GPx and a decreased level of MDA were observed. A time point study is needed to identify the causality of changes of these biomarkers to identify if it is a direct antioxidant effect of Leonurine. Nonetheless, we propose that an in vivo therapeutic effect of Leonurine is strongly related to antioxidant effect by enhancing the endogenous antioxidant capacity and therefore alleviates the oxidative stress during ischemic stroke.
Dysfunctional mitochondria produce more ROS, and a feed-forward loop is set up whereby ROS-mediated oxidative damage to mitochondria favors more ROS generation, resulting in a vicious cycle. This phenomenon was shown in our study that as compared with the control group, H_2O_2-treated mitochondria produced much more free radicals. We demonstrated for the first time that Leonurine significantly reduced the mitochondrial ROS production in both physiological and H_2O_2-treated condition in a dose-dependent manner. The reduction of physiological mitochondrial ROS generation indicates that pretreatment with Leonurine provides a protective barrier to oxidative stress. A reduction of mitochondrial ROS production or mitochondrial oxidative stress could slow down the progression of ischemic stroke.

Several enzymes of ETC were shown to be inhibited by ROS. ROS also attack the mitochondrial membrane directly to cause the membrane damage. These factors lead to depletion of ATP. In our study, ATP biosynthesis was shown to be suppressed when mitochondria were treated with H_2O_2. In both physiological and H_2O_2-treated condition, Leonurine further suppressed the ATP biosynthesis dose-dependently, suggesting that Leonurine might induce mitochondrial metabolic arrest, reducing ATP production to minimal. It has been reported that animals undergoing hibernation had metabolic depression on mitochondrial ATP biosynthesis\(^{23}\) and it is known to be cytoprotective against various hypoxic and ischemic insults, enabling the organism to survive under deprived conditions over a period of time.\(^{26}\)

Mitochondrial respiration is important for determining the coupling state of oxidative phosphorylation in the mitochondria, thus regulating the level of mitochondrial metabolic efficiency. The RCR (state 3 respiration rate/state 4 respiration rate) indicates the tightness of the coupling respiration and phosphorylation. In vivo in experimental work, we showed Leonurine treatment enhanced mitochondrial oxygen consumption in the sham group with no change of RCR value, suggesting that pharmacological preconditioning by Leonurine could enhance the function of mitochondria. We also found that left cortical mitochondrial oxygen consumption (state 3 respiration) was greatly suppressed 3 hours after MCAO. In consistent with previous work,\(^{22}\) the lowest RCR obtained from the vehicle group, indicating that mitochondrial dysfunction resulting after permanent MCAO. Leonurine rescued the mitochondria by improving the oxygen consumption to the level that is almost comparable to the sham untreated group. The RCR value from the stroke Leonurine-treated group was also increased, suggesting that Leonurine could prevent the mitochondrial dysfunction from oxidative stress.

To the best of our knowledge, this is the first demonstration of the therapeutic potential of Leonurine on permanent MCAO. We revealed that pretreatment of Leonurine at 60 mg/kg per day for 1 week protects rats from stroke insult by permanent MCAO through reduction of infarct volume and improvement of neurological deficit in stroke groups. Leonurine protects the cells from neuronal injury, at least through the intervention with oxidative stress. In addition, Leonurine modulates mitochondrial function by inhibiting ROS production and ATP biosynthesis. Animal studies also demonstrated that the mitochondrial dysfunction was rescued by Leonurine treatment. This study shows that pretreatment of Leonurine has neuroprotective effects and carries a potential to be a preventive measure for a population with a higher risk of having stroke. We also believe that treatment of Leonurine might be beneficial to stroke patients against a second occurrence of stroke because endogenous antioxidant capacity might be enhanced by Leonurine treatment. However, it is unknown whether this neuroprotective effect of Leonurine is based on direct antioxidant effect. The specific mechanism of Leonurine involved in mediating precondition requires further investigation. Above all, we conclude that Leonurine has neuroprotective effects and carries a potential for stroke prevention.

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**Disclosures**

None.

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