Synergetic Neuroprotection of Normobaric Oxygenation and Ethanol in Ischemic Stroke Through Improved Oxidative Mechanism

Xiaokun Geng, MD*; Paul Fu, BS*; Xuming Ji, MD, PhD; Changya Peng, MD; Vance Fredrickson, BS; Christopher Sy, BS; Ran Meng, MD, PhD; Feng Ling, MD, PhD; Huishan Du, MD; Xiaomu Tan, MD; Maik Hüttemann, PhD; Murali Guthikonda, MD; Yuchuan Ding, MD, PhD

Background and Purpose—Normobaric oxygenation (NBO) and ethanol both provide neuroprotection in stroke. We evaluated the enhanced neuroprotective effect of combining these 2 treatments in a rat stroke model.

Methods—Sprague-Dawley rats were subjected to middle cerebral artery occlusion for 2 hours. Reperfusion was then established and followed by treatment with either (1) an intraperitoneal injection of ethanol (1.0 g/kg), (2) NBO treatment (2-hour duration), or (3) NBO plus ethanol. The extent of brain injury was determined by infarct volume and motor performance. Oxidative metabolism was determined by ADP/ATP ratios, reactive oxygen species levels, nicotinamide adenine dinucleotide phosphate oxidase activity, and pyruvate dehydrogenase activity. Protein expression of major nicotinamide adenine dinucleotide phosphate oxidase subunits (p47-phox, gp91-phox, and p67-phox) and the enzyme pyruvate dehydrogenase was evaluated through Western immunoblotting.

Results—NBO and ethanol monotherapies each demonstrated reductions as compared to stroke without treatment in infarct volume (36.7% and 37.9% vs 48.4%) and neurological deficits (score of 6.4 and 6.5 vs 8.4); however, the greatest neuroprotection (18.8% of infarct volume and 4.4 neurological deficit) was found in animals treated with combination therapy. This neuroprotection was associated with the largest reductions in ADP/ATP ratios, reactive oxygen species levels, and nicotinamide adenine dinucleotide phosphate oxidase activity, and the largest increase in pyruvate dehydrogenase activity.

Conclusions—Combination therapy with NBO and ethanol enhances the neuroprotective effect produced by each therapy alone. The mechanism behind this synergistic action is related to changes in cellular metabolism after ischemia reperfusion. NBO plus ethanol is attractive for clinical study because of its ease of use, tolerability, and tremendous neuroprotective potential in stroke. (Stroke. 2013;44:1418-1425.)

Key Words: aerobic glucose metabolism ■ ATP ■ combination therapy ■ ischemia/reperfusion ■ reactive oxygen species
maximum legal driving limit of 0.08%–0.10%) produces strong neuroprotection in rats subjected to a 2-hour middle cerebral artery occlusion (MCAO). Because both NBO and ethanol are easily delivered into the ischemic brain through the collateral circulation and diffusion through the blood–brain barrier, concurrent application of ethanol in NBO-treated ischemic rats may enhance neuroprotection.

Ischemia–reperfusion injury in acute stroke disrupts energy production by impairing the metabolic state of neural cells and interrupting mitochondrial activity. Aerobic glucose metabolism begins in the cytoplasm with glycolysis, and ends in the mitochondria with oxidative phosphorylation, which produces the majority of cellular ATP. During ischemia, oxygen deprivation impairs oxidative phosphorylation and significantly limits ATP production, leading to an overproduction of reactive oxygen species (ROS) during reperfusion. Activation of nicotinamide adenine dinucleotide phosphate oxidase (NOX), especially during reperfusion, also contributes to the generation of ROS. ROS further impairs the expression of pyruvate dehydrogenase (PDH), which serves as the critical link between glycolysis (anaerobic metabolism) and the tricarboxylic acid cycle by catalyzing the important transition step of pyruvate to acetyl coenzyme A. The down-regulation of PDH potentiates metabolic stress.

To assess the synergistic effects of NBO combined with ethanol treatment, we evaluated the degree of neuroprotection by determining infarct volume and functional outcomes after ischemia–reperfusion injury. Metabolic mechanisms were determined by alterations in the ADP/ATP ratio, ROS generation, NOX activation, and PDH expression.

Methods

All experimental procedures were approved by the Institutional Animal Investigation Committee of Wayne State University and were in accordance with the National Institutes of Health guidelines for care and use of laboratory animals. A total of 112 adult male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) were used. Rats were randomly divided into 5 groups, including a sham-operation group, and 4 stroke groups consisting of 1 stroke without therapy and 3 treatment groups. Within the treatment groups, rats were assigned to receive either an intraperitoneal injection of ethanol (1.0 g/kg) at reperfusion, NBO (95% O2) administered immediately after reperfusion for 2 hours, or a combination of NBO and ethanol. All studies were performed in a blinded and randomized manner.

Focal Cerebral Ischemia

MCAO was induced for 2 hours using the intraluminal filament model. Blood P<sub>Ar</sub> and P<sub>ca</sub>, mean arterial pressure, as well as rectal and brain temperature were monitored throughout the procedure. The rectal temperature was maintained at 36.5°C to 37.5°C using a circulating heating pad and a heating lamp.

NBO Treatment

After 2 hours of MCAO, reperfusion was achieved by withdrawal of the intraluminal filament. Ischemic animals were then exposed to 95% O2 for a duration of 2 hours in a sealed chamber (50×25×25 cm). The oxygen concentration and flow rate (2 L/min) were maintained using an oxygen controller (PRO-OX110; Reming Bioinstruments Co., Redfield, NY). Carbon dioxide was continuously removed by soda lime (Sigma, USA) placed at the bottom of the chamber.

NBO and Ethanol in Combination

Rats in stroke groups were given 3 mL of either ethanol (1.0 g/kg diluted in saline to 3.0 mL) or normal saline by intraperitoneal injection at the initiation of reperfusion after the 2 hours of MCAO. In our previous study, ethanol at a dose of 0.5 g/kg did not effectively produce neuroprotection, whereas the 1.0 g/kg dose created only a mild neuroprotective effect as compared to the 1.5 g/kg dose. Therefore, ethanol was used at the minimally effective dose (1.0 g/kg) to determine if a synergistic effect could be found by combining ethanol and NBO therapies.

Neurobehavioral Functional Testing

Rats were examined based on the scoring system proposed by Belayev et al 13 before surgery, at 2 hours after MCAO, and at 24 hours after reperfusion. Greater scores indicated a more severe deficit.

Cerebral Infarct Volume

Forty-eight hours after reperfusion, 8 rats per stroke group (n=32) were anesthetized and euthanized by decapitation. Coronal brain sections for 2-mm thickness were taken for treatment with 2,3,5-triphenyltetrazolium chloride (Sigma, USA). 9,12 To minimize error introduced by edema, an indirect method was used for calculating infarct volume by taking it as a percentage of total ipsilateral brain volume. 6

Metabolic Activity and Protein Expression

Metabolic activity was analyzed in animals from each of the 5 groups (8 rats per group) euthanized at 3 (n=40) and 24 hours (n=40) after reperfusion. Brain samples were taken from the frontoparietal cortex and striatum as supplied by the MCA.

ADP/ATP Ratio Assay

The ADP/ATP ratio assay was performed using the BioVision Assay Kit (CA) as previously described by us. 14 Luminescence was detected using a DTX-880 Multimode Detector for ATP and ADP levels. ADP/ATP ratios were subsequently calculated.

ROS Production

The method for detection of ROS, described previously, 13 tests for H<sub>O2</sub> levels with hydrogen peroxidase linked to a fluorescent compound. Homogenized brain samples taken from the animals were diluted to 10 mg/mL based on the protein concentration bicinchonic acid-BCA method. After 30 minutes of incubation, H<sub>O2</sub> levels in brain homogenates were determined using 50 μmol/L Amplex red, 0.1 U/mL horseradish peroxidase, and respiratory substrates (4 mmol/L pyruvate, 2 mmol/L malate, 2 mmol/L glutamate, and 0.8 mmol/L complex V inhibitor oligomycin) at 37°C on a DTX-880 Multimode Detector.

NOX Activity

NOX activity was determined as described previously. 13 Brain homogenized samples containing phenylmethylsulfonyl fluoride and a protease inhibitor cocktail (Thermo; 20 μL) were added to a 96-well luminescence plate containing 6.25 μmol/L of lucigenin. The reaction was initiated by the addition of nicotinamide adenine dinucleotide phosphate (100 μmol/L). NOX activity was calculated by the change in luminescence recorded by the DTX-880 Multimode Detector.

PDH Enzyme Activity

PDH enzyme activity was determined using the PDH activity assay kit (Abcam, Cambridge, MA). 13 Homogenized brain samples were loaded on a plate and incubated in the Assay Solution. The OD<sub>490</sub> was measured at 20-second intervals for 30 minutes by a DTX-880 Multimode Detector (Beckman Coulter). The slope of the curve was calculated and reported.
Protein Expression of NOX Subunits and PDH

Western blot analysis was used to assess protein expression of NOX subunits (p47^{phox}, gp91^{phox}, and p67^{phox}) and PDH as described previously, using the selected primary antibodies (polyclonal goat anti-gp91^{phox} 1:2000, polyclonal goat anti-p47^{phox} 1:1000, polyclonal goat anti-p67^{phox} 1:1000, and polyclonal rabbit anti-PDH 1:250; Santa Cruz Biotechnology, Inc.). Equal protein loading was confirmed and adjusted using β-actin (goat polyclonal anti-β-actin antibody, 1:1000; Santa Cruz Biotechnology, Inc.). Targeted antigens were visualized using standard chemical luminescence methods (Amersham ECL; GE Healthcare BioSciences, Piscataway, NJ). Quantification of relative target protein expression was performed using the program ImageJ 1.42 (National Institutes of Health).

Statistical Analysis

Statistical analysis was performed with SPSS for Windows, version 17.0 (SPSS, Inc.). The differences among groups were assessed using 1-way ANOVA with a significance level at \( P<0.05 \). Post hoc comparison among groups was further performed using the least significant difference method.

Results

Physiological Parameters

There were no significant differences with respect to blood pH and \( P_{aco_2} \) and mean arterial pressure among the groups (Table). NBO treatment significantly \( (P<0.01) \) elevated \( P_{ao_2} \) levels (>400 mm Hg) compared with stroke without treatment (129 mm Hg). Blood glucose levels were significantly \( (P<0.01) \) increased after MCA occlusion, but no difference was found among groups. Body temperature was maintained \( \approx 37^\circ C \).

Infarct Volume and Neurological Deficit

At 48 hours after reperfusion, both ethanol (37.9±3.4\%) and NBO (36.7±4.1\%) produced a small but significant \( (P<0.05) \) decrease in infarct volume when compared with the stroke without treatment group (48.4±4.2\%; \( F_{[3, 28]}=12.0; P<0.01; \) Figure 1A). This decrease in infarction volume was largely \( (P<0.01) \) enhanced by combination therapy with NBO and ethanol (18.8±2.1\%).

After 2 hours of MCAO, ischemic rats demonstrated readily apparent neurological deficits (higher score; data not shown). At 24 hours after reperfusion, a significantly high score (8.4±0.7) was observed in the nontreatment group (\( F_{[1, 28]}=8.1; P>0.001; \) Figure 1B), which was significantly reduced by both ethanol (6.5±0.7) and NBO (6.4±0.6) monotherapies \( (P<0.05) \). However, the NBO+ethanol combination therapy exhibited the greatest reduction (4.4±0.3) in neurological deficits \( (P<0.01) \).

Energy Production Determined by ADP/ATP Ratio

The metabolic viability of cerebral tissue was assessed by measuring the ADP/ATP ratio. When compared with the sham-operated group, the ADP/ATP ratio was significantly elevated at 3 hours (\( F_{[4, 35]}=12.2; P<0.01 \)) after reperfusion in all ischemic groups and was further elevated at 24 hours (\( F_{[4, 35]}=79.1; P<0.01 \)) after reperfusion in these groups, indicating an impaired energy balance (Figure 2). Compared with stroke without therapy, both NBO and ethanol significantly \( (P<0.01) \) decreased the ADP/ATP ratio, and this reduction was enhanced by the combinational therapy

\( (P<0.01) \) at both time points. This decrease in the ADP/ATP ratio indicates an efficient preservation of ATP levels by NBO+ethanol treatment after ischemia/reperfusion injury.
Oxidative Stress Determined by the Levels of ROS

In ischemic rats, ROS levels were significantly increased as early as 3 hours (F[4,30]=31.3; P<0.01) and remained elevated at 24 hours (F[4,30]=4.1; P<0.05) after reperfusion (Figure 3). Although a significant decrease in ROS levels was observed in monotherapy groups at 3 and 24 hours, combinational therapy most effectively reduced ROS levels at both time points (P<0.01).

NOX Activity and Protein Subunit Expression

Across all stroke groups, an increase in NOX activity was observed at 3 (F[4,30]=3.8; P<0.05) and 24 hours (F[4,30]=10.4; P<0.01) after reperfusion compared with sham-operated animals (P<0.05; Figure 4A). Combination treatment with NBO+ethanol most effectively (P<0.05) decreased NOX activity compared with either monotherapy at both time points. NBO treatment alone did not significantly reduce NOX activity, whereas ethanol by itself reduced NOX activity only at 24 hours. In all ischemic groups, there was a significant increase in protein levels for gp91phox at 3 (F[4,30]=9.8; P<0.01) and 24 hours (F[4,30]=7.3; P<0.01; Figure 4B), for p47phox at 3 (F[4,30]=8.3; P<0.01) and 24 hours (F[4,30]=3.6; P<0.05; Figure 4C), and for p67phox at 3 (F[4,30]=10.9; P<0.01) and 24 hours (F[4,30]=8.3; P<0.01; Figure 4D), respectively, compared with the sham-operated control group. In monotherapy groups, small reductions of p47phox, p67phox, and gp91phox proteins were observed at 3 and 24 hours, whereas combination treatment induced a much greater reduction in all NOX subunits (P<0.01). These findings indicate that both ethanol and NBO exert an inhibitory effect on NOX subunit expression, and that this effect is significantly enhanced with combinational therapy.

PDH Activity and Protein Expression

Compared with the sham-operated group, PDH enzyme activity was significantly reduced in ischemic rats after reperfusion at 3 (F[4,30]=12.1; P<0.01) and 24 hours (F[4,30]=14.8; P<0.01; Figure 5A). This reduction in PDH activity was only slightly reversed by ethanol or NBO monotherapy.

However, combinational therapy largely reversed this reduction (P<0.01). Similarly, Western blot analyses for the stroke-without-treatment group demonstrated a significant reduction in PDH protein expression at 3 (F[4,30]=3.9; P<0.01) and 24 hours (F[4,30]=2.7; P<0.05) after reperfusion compared with sham-operated group (Figure 5B). Individual therapies of ethanol or NBO did not significantly reverse this reduction in PDH expression at either time point. However, combination treatment resulted in a significant and drastic increase in PDH expression at both 3 and 24 hours (P<0.01).

Discussion

In the present study, we demonstrated that combinational therapy using NBO plus ethanol produces enhanced neuroprotection compared with either treatment alone. This was evident from the maximal reduction in infarct volume and neurological deficit of the combinational therapy; its significant decreases in the ADP/ATP ratio, ROS generation, and NOX activation; and its simultaneous enhancement of PDH activity and protein expression. All of this evidence points to a synergistic neuroprotective effect of combination treatment, produced with relatively low doses of both ethanol and NBO. Neuroprotection produced by combinational therapy is described as synergistic because both NBO and ethanol exert significant influences on oxidative metabolism and the reduction in infarct volume of ethanol plus NBO was much greater than the summation of individual treatments (ethanol + NBO: 61.2% versus ethanol: 21.7% and NBO: 24.2%). Combination therapy was also the only treatment that significantly and dramatically decreased NOX, with the change being well above an additive effect. PDH activity and protein expression also followed a similar pattern.

Epidemiological studies have suggested a role for ethanol as a potential neuroprotectant in stroke. We previously demonstrated that 1.0 g/kg ethanol (which correlates with a blood alcohol concentration of 56 mg/dL, below legal driving limit of 80 mg/dL) produces a mild neuroprotective effect compared with a higher dose (1.5 g/kg, which correlates with a blood alcohol concentration of 89 mg/dL). Previous studies have demonstrated that NBO administration 30 minutes after MCA occlusion for up to a duration of 5 hours produced only
marginal neuroprotective effect that was significantly eclipsed by HBO treatment.21,22 Thus, NBO therapy 2 hours after MCA occlusion in the present study is considered delayed, which only induced minimal neuroprotection. By combining 2 low-dose therapies with similar mechanisms of action on oxidative metabolism, we hoped to increase target effects and reduce adverse reactions as well. Ethanol has already been safely administered as a monotherapy in the clinical setting as an antidote to methanol and other toxic alcohols.23 An important safety concern with oxygen therapy is the potential for increased ROS production, which was especially problematic in HBO treatment.22,24 However, recent studies suggest that the benefits of NBO may actually exceed the risk of enhanced oxidative stress,4,7 and this risk may be further reduced in transient focal cerebral ischemia.25 Tatarkova et al26 demonstrated that prolonged NBO treatment is accompanied by a significant increase in mitochondrial oxidative damage. This is consistent with findings that prolonged exposure to hyperoxia can activate apoptosis.27 Thus, by using NBO for a short duration and combining it with a low dose of ethanol, we looked to produce strong neuroprotection, while minimizing toxic side effects as determined by low ROS production and markers of oxidative damage.

Disruption of cell metabolism and mitochondrial dysfunction have long been associated with increases in oxidative stress through the overproduction of ROS, including superoxide radicals and peroxides. These ROS products, in turn, play a significant role in brain ischemia/reperfusion injury,28 leading to the exacerbation of brain edema, postischemic hemorrhage, and neuronal death.25,29 The beneficial effect of combination therapy on reduced oxidative stress, in our study, may be attributed to its effect on NOX and PDH, which are important enzymes related to ROS production and oxidative metabolism. NOX, a membrane-bound enzyme complex, has been shown to exacerbate ischemia/reperfusion injury30 and is a well-known major source of ROS generation in stroke. NOX is a multicomponent cytoplasmic enzyme (p47phox, p67phox, p40phox, and Rac2) that, on phosphorylation of the p47phox subunit,31 forms a complex and translocates to the plasma membrane to dock with specific plasma membrane subunits such as p91phox. The enzymatic core of NOX comprises the gp91phox,31 and a catalysis occurs in this subunit.10 Because NOX subunits play a major role in formation of its complex, down-regulation of these subunits lead to inhibited NOX

Figure 4. A, Nicotinamide adenine dinucleotide phosphate oxidase (NOX) activity was significantly increased in stroke (*P<0.01) compared with sham-operation control referenced as 1. Ethanol (EtOH), but not normobaric oxygenation (NBO), reduced enzyme activity at 24 hours (#P<0.05). Combination therapy, however, significantly reduced NOX activity at both the 3- and 24-hour time points (##P<0.01). B, Subunit gp91phox protein expression was elevated after stroke (*P<0.01). EtOH (#P<0.05) and NBO slightly reduced protein expression at 3 and 24 hours. This reduction in gp91phox was enhanced by combination therapy (##P<0.01). C, p47phox protein expression was significantly elevated after stroke (*P<0.01). Although both EtOH and NBO significantly reduced protein expression at 3 and 24 hours (#P<0.05), the reduction in p47phox was enhanced by combination therapy (##P<0.01). D, p67phox protein expression was significantly elevated after stroke (*P<0.01). EtOH (#P<0.05) or NBO reduced protein expression at 3 and 24 hours. However, combination therapy (##P<0.01) largely enhanced this reduction.
The present study demonstrates that although NBO and ethanol monotherapies both seem helpful in reducing oxidative stress in ischemia/reperfusion injury, their combination produces substantially greater decrease in NOX activity and protein expression. The concomitant reduction of ROS products suggests that the decrease in NOX induced by combination therapy is at least partially responsible.

PDH, which is located in the mitochondrial matrix and is composed of 3 major subunits (E1, E2, and E3), plays a key role in aerobic energy metabolism. PDH serves as the critical linking enzyme between glycolysis (anaerobic metabolism), the tricarboxylic acid cycle, and oxidative phosphorylation (aerobic metabolism) by catalyzing the oxidative decarboxylation of pyruvate to acetyl coenzyme A. Previous studies have shown that ischemia-reperfusion injury impairs PDH enzyme activity even after the replacement of glucose and oxygen, leading to changes in mitochondrial respiration and failure of energy production. Our findings demonstrate that combination therapy is most efficacious in attenuating the impaired PDH activity and protein expression, thus creating a neuroprotective effect in stroke. This raises the possibility that NBO and ethanol treatments help to facilitate aerobic pathways of energy production, as shown by the reduction in ADP/ATP ratios of the combination therapy. This observation is consistent with previous findings that acute ethanol administration decreases ADP/ATP ratios and increases cell viability. Previous studies have also shown that ROS mediates inactivation of PDH and that the PDH complex is a target of oxidative stress. It is likely that the up-regulation of PDH activity, which can be attributed to decreased ROS by ethanol, helps to preserve aerobic metabolism further enhanced by NBO. This, in turn, stabilizes cellular metabolism and mitochondrial function, preventing the depletion of cellular ATP and reducing subsequent brain damage.

Stroke is a complex disease involving multiple deleterious processes that occur during the ischemic and postischemic period. Previous unsuccessful monotherapeutic approaches may have failed because they did not adequately address all the modalities of such a complex injury. In contrast, combinational therapy can target alternate neuroprotective pathways or synergistically affect a single mechanism, thereby producing more promising results. From our study, ROS production seems to play a central role in ischemia/reperfusion injury, and subsequent reduction of this oxidative stress is key to neuroprotection. However, the data also show that combination therapy targets multiple sites, including both upstream and downstream ROS production. It also synergistically improves

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**Figure 5.** A, Pyruvate dehydrogenase (PDH) activity was significantly decreased (*P<0.05) after reperfusion at 3 and 24 hours compared with sham-operation control. Only ethanol (EtOH) monotherapy significantly raised PDH activity at one time point, 3 hours after reperfusion (*P<0.05). However, combination therapy most effectively raised PDH activity at both 3 and 24 hours (**P<0.01). B, Similarly, ischemia followed by 3 and 24 hours of reperfusion significantly (*P<0.05) decreased PDH expression. Both monotherapies slightly increased PDH levels, whereas combination therapy was found to significantly (**P<0.01) increase PDH expression at 3 and 24 hours.

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**Table.** Physiological Parameters

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EtOH indicates ethanol; MAP, mean arterial pressure; MCAO, middle cerebral artery occlusion; and NBO, normobaric oxygenation.

* NBO elevated P<2 levels to ≈400 mm Hg, ANOVA indicates P<0.01 compared with stroke and stroke and ETOH groups.
oxidative metabolism by increasing the activity of the key limiting enzyme PDH and providing oxygen supply for aerobic metabolism. Therefore, ethanol and NBO in combination would likely have stronger therapeutic effects than antioxidant treatment alone. In addition, several other mechanisms could be the source of neuroprotection demonstrated by combinational therapy. In a recent study,\(^40\) NBO administration after reperfusion was shown to significantly reduce expression of caspase-3 and apoptosis-inducing factor in the rat stroke model. These 2 proteins have been shown to play an important role in signaling for cell death.\(^40,41\) Current work in our laboratory (unpublished data) has also shown that ethanol has similar effects in the down-regulation of apoptotic proteins, and this decrease in protein expression is directly related to a reduction in apoptosis. Another possible neuroprotective mechanism is through improvements in the integrity of the blood–brain barrier. Liu et al.\(^42\) demonstrated that NBO attenuates blood–brain barrier disruption in ischemia-reperfusion injury, by inhibiting matrix metalloproteinase-9 occludin degradation. Our work\(^43\) shows that ethanol produces a similar effect in the ischemic brain, ameliorating blood–brain barrier disruption by decreasing expression of matrix metalloproteinases and aquaporins.

In summary, combination therapy of ethanol and NBO is inexpensive, well tolerated, and widely available; it produces a powerful neuroprotective effect even at low doses in ischemia-reperfusion injury. As such, combination therapy has high translational significance and is a promising therapeutic target for acute clinical stroke therapy. However, further studies are still required to better understand this novel treatment. An interesting future topic of our laboratory is to determine whether a combinational therapy continues to provide effective neuroprotection in ischemia reperfusion of longer duration, and even in permanent strokes. Another point of focus will be the identification of the metabolic effects of combination therapy beyond the 24-hour time point. Finally, additional mechanistic studies will be needed to help clarify the synergistic neuroprotection demonstrated by ethanol and NBO.

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### Disclosures

None.

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