

Hepatoprotective effect of silver nanoparticles synthesized using aqueous leaf extract of *Rhizophora apiculata*

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Background: Liver plays a vital role in the elimination of xenobiotics that can induce hepatotoxicity in living organisms. Silver nanoparticles have evolved recently as an alternative in various industries and are used for their biomedical applications. *Rhizophora apiculata* is a least studied mangrove-based plant that has been used in the traditional medicine of Southeast Asia for its healing properties. It is a well-known fact that the generation of free radicals has been associated with oxidative stress.

Methods: Hence, in this study we used carbon tetrachloride as a hepatotoxin to induce liver damage. The protective effects of silver nanoparticles synthesized using *Rhizophora apiculata* on hepatotoxin-induced liver damage in experimental mice were assessed.

Results: The results of the assessment indicate that silver nanoparticles were effective in protecting the liver from damages induced by carbon tetrachloride.

Conclusion: Among existing literature, this is the first ever approach for hepatoprotective effect of nanoparticles derived using plant extract from mangrove ecosystem.

Keywords: mangroves, *Rhizophora apiculata*, silver nanoparticles, carbon tetrachloride, hepatotoxicity

Introduction

The liver plays an important role in metabolism and detoxification of compounds which enter the body and may cause hepatic injury, leading to life-threatening diseases.¹ Therefore, major toxicological problems associated with several diseases have been centered around the effects on the liver.² Usually, liver cells are affected by hepatotoxic agents through the induction of oxidative damage.³ Drugs of both synthetic and natural origin are available for treatment of liver diseases.⁴ Natural remedies have long been used for treatment of liver diseases. Based on this, protective effects of plant-based herbal medicines against drug-induced toxicity have reached paramount importance recently.⁵

Rhizophora apiculata is a member of the Rhizophoraceae family of the south-east Asian mangrove ecosystem.⁶ Members of *Rhizophora* family have been known to possess several biomedical compounds.⁷ *R. apiculata* has not been studied in depth regarding its therapeutic values, although reports suggest that they possess some biological activities when used in diseases such as cancer.⁸ To the best of our knowledge, among available literature, there are no reports yet on the hepatoprotective activity of *R. apiculata*. Therefore, the leaf extract and the extract-derived nanoparticles have been used in this study to analyze their protective effects on the liver.

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Carbon tetrachloride (CCl₄) is a xenobiotic released into water as waste from several industries, thereby leading to hepatotoxicity when living organisms are exposed to it.⁹ It is often used to induce liver disorders in various models for the screening of hepatoprotective agents.¹⁰ In the studies that involve animal models, particularly those that study the effects on the liver, CCl₄ was transformed into other forms of free radicals, leading to lipid peroxidation. This may therefore result in cellular necrosis.¹¹ Hence, in this study, we used CCl₄ as the hepatotoxin to analyze the effects on the liver.

Free radicals can also be generated by normal physiological conditions.¹² However, when there is an imbalance in ROS and free radical scavenging, it results in tissue damage and cell death.¹³ Elevated levels of ROS can determine the occurrence of oxidative stress.¹⁴ ROS can therefore have a profound effect on several infections induced by chemical and biological means.¹⁵ Therefore, we intended to test the ROS and oxidative stress-related biochemical parameters to analyze the hepatoprotective effect of the nanoparticles.

Silver nanoparticles (AgNPs) have been exploited for several therapeutic purposes because of its properties such as limited toxicity, increased biodegradability, and bioavailability.^{16,17} They are known to interact easily with biological systems because of their small size.¹⁸ AgNPs can be synthesized via various routes, among which, green synthesis is considered to be the need of the hour, since it possesses several advantages compared to chemical synthesis.^{19–21} Biosynthesis of AgNPs has been performed using several biological sources and such nanoparticles have been used in various industries.²² But recently, the synthesis of AgNPs using plants has become the most preferred method for biosynthesis of nanoparticles.²³ AgNPs biosynthesized using *Andrographis paniculata* have been known to possess hepatoprotective properties.²⁴ However, there are no reports on hepatoprotective activity of mangrove-derived AgNPs.

Taking this as an initiative, we intended to study the hepatoprotective activity of AgNPs in CCl₄ induced mouse model. A plant leaf extract of the mangrove *R. apiculata* and AgNPs derived using the same plant leaf extract (shade-dried leaf extract as a reducing agent) were used as test agents. The procedure for synthesis and characterization of AgNPs using *R. apiculata* has been published earlier by our group.²⁵

Materials and method

Characterization of AgNPs

UV-visible spectroscopy, Fourier transform infra-red spectroscopy and transmission electron microscopy were used

for characterization of shade-dried *R. apiculata* leaf extract-derived AgNPs in our previously published report.²⁵ In the present study, the AgNPs were further characterized by scanning electron microscopy (SEM), energy dispersive X-ray (EDX) analysis, inductively coupled plasma-optical emission spectrometry (ICP-OES), and X-ray photoelectron spectroscopy (XPS). SEM and EDX analysis were performed using JEOL-JSM 6390 instrument. Concentration of AgNPs was determined by ICP-OES analysis (Perkin Elmer Optima 5300 DV model, PerkinElmer Inc., Waltham, MA, USA). XPS analysis was performed with Omicron ESCA Probe spectrometer.

Chemicals and laboratory animals

Silymarin and CCl₄ were obtained from Ranbaxy Laboratories and Qualigens respectively. Fifty-four male Swiss albino mice weighing around 30–35 g, bought from Kerala Veterinary and animal Sciences University, Thrissur, Kerala, India, were used for the study. The animals were confined in polypropylene cages and fed with standard pellet and water ad libitum. A temperature of 23°C–25°C and humidity of 50%–60% were maintained throughout the experiment with a 12-hour light–dark cycle. Standard pellet diet was purchased from Sai Enterprise, Chennai, India. Permission for the experiment was obtained from the Institutional Animal Ethics Committee, and CPCSEA regulations were followed during the entire course of the study (Ethics Committee number: BDU/IAEC/42/2013/09.04.2013).

Experimental plan

Fifty-four Swiss albino male mice were divided into nine groups. Each group consisted of six mice. Group I animals were normal. Animals intoxicated with CCl₄ intraperitoneally (1:1 with olive oil, 0.5 mL/kg of body weight [BW]) for 2 days at the start of the experiment were Group II (control). Group III (low dose [LD] – 1.75 mg/kg BW), Group IV (medium dose [MD] – 2.5 mg/kg BW), and Group V (high dose [HD] – 3.25 mg/kg BW) were CCl₄-intoxicated mice treated with the specified doses of aqueous leaf extract of *R. apiculata*. Groups VI (1.75 mg/kg BW – LD), VII (2.5 mg/kg BW – MD), and VIII (3.25 mg/kg BW – HD) were CCl₄-intoxicated mice treated with the specified doses of *R. apiculata*-derived AgNPs. Group IX animals were treated with silymarin (standard drug, 20 mg/kg BW) after intoxication with CCl₄. The groups involved in the study are explained in Table 1. Treatment was initiated orally from day 3 of the induction of hepatotoxicity. The

Table I Experimental groups involved in analysis of hepatoprotective activity of silver nanoparticles

Groups (6 animals each)	Carbon tetrachloride mixed 1:1 with olive oil	<i>R. apiculata</i> leaf extract	<i>R. apiculata</i> -derived silver nanoparticles	Silymarin
I	-	-	-	-
II	0.5 mL/kg body weight (BW)	-	-	-
III	0.5 mL/kg BW	Low dose - 1.75 mg/kg BW	-	-
IV	0.5 mL/kg BW	Medium dose - 2.5 mg/kg BW	-	-
V	0.5 mL/kg BW	High dose - 3.25 mg/kg BW	-	-
VI	0.5 mL/kg BW	-	Low dose - 1.75 mg/kg BW	-
VII	0.5 mL/kg BW	-	Medium dose - 2.5 mg/kg BW	-
VIII	0.5 mL/kg BW	-	High dose - 3.25 mg/kg BW	-
IX	0.5 mL/kg BW	-	-	20 mg/kg BW

experiment was continued for 7 more days. Serum was collected at the end of the experiment by euthanizing the animals. Clear serum obtained after centrifuging at 2,500 rpm for 15 minutes was used for further analyses.

Biochemical parameters

Liver function test was performed by analyzing markers such as SGOT, SGPT, and ALP.²⁶ Levels of enzymatic and non-enzymatic antioxidants such as GPx and GSH were estimated.²⁷ Cellular metabolite LDH was studied to analyze tissue injury.²⁸ MDA was studied to analyze lipid peroxidation.²⁹

Histopathology

Livers were collected after euthanizing the mice at the end of the experimental period. After processing with formalin and staining with H&E, the histopathological changes in the livers were observed under a microscope at 40× using paraffin sections of 5 μm thickness.³⁰

Statistics

Results obtained were expressed as mean ± SD. SPSS version 17 was applied to perform one-way ANOVA.³¹

Results and discussion

Figure 1 shows the SEM micrograph of the biosynthesized AgNPs. Silver was the foremost element (69.8%) in comparison with chlorine (21.81%) and oxygen (8.39%), as observed by EDX spectrum. The weaker peaks of O and Cl in the EDX spectrum are probably due to X-ray emissions from proteins or sugars.³² No peaks for compounds of

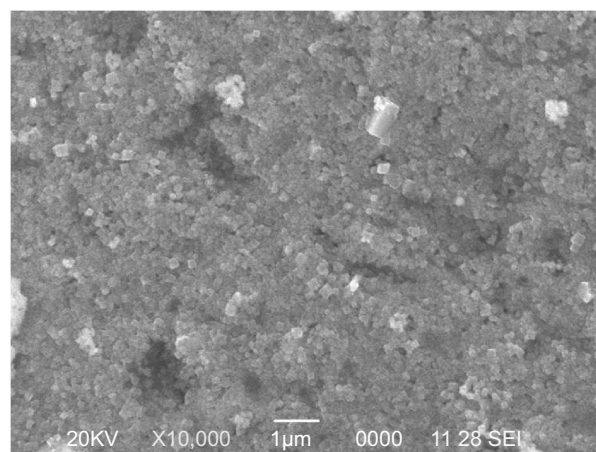


Figure 1 Scanning electron microscopy analysis of the synthesized nanoparticles.

silver were observed (Figure 2). This confirms that silver nitrate was reduced to AgNPs. The spectrum around 3 KeV stands as a strong signal for the presence of AgNPs due to surface plasmon resonance.³³ The total concentration of AgNPs, as calculated by ICP-OES, a widely used method to detect metal ions, was 19.51 mg/L.³⁴ Strong peaks of C1s, O1s, and Ag3d core levels were found in XPS analysis. There was a major C1s peak at 286.8 eV. Considering the carbon shift from 284.8 eV, the O1s value was centered at 532 eV. Ag3d_{5/2} and Ag3d_{3/2} peaks occurred at binding energies of 367.8 and 374.0 eV, respectively (Figure 3). This suggests the oxidation state at Ag.³⁵ The Auger core level value stood at 358.5 eV. The metallic state of Ag was further confirmed by Wagner plot. The Auger parameter between 726.00 and 726.40 corresponds to the state of pure silver. Values in the range of 722.80–724.80

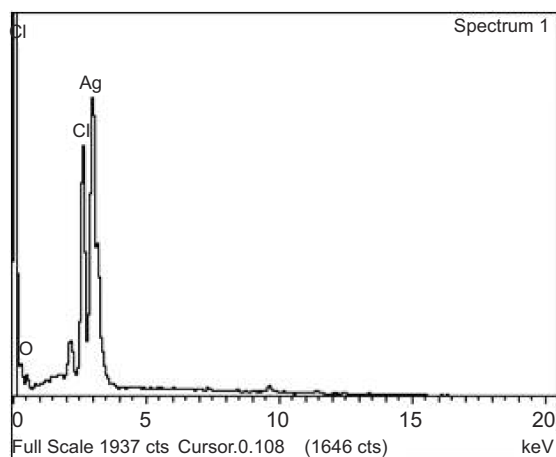


Figure 2 Energy dispersive X-ray analysis of the synthesized nanoparticles.

correspond to the presence of silver salts. Auger parameter was calculated by the sum of Auger core value (358.5 eV) and the $Ag3d_{5/2}$ value (367.8 eV). This resulted in a sum of 726.3, the Auger parameter specific for metallic Ag.³⁶ This protocol again suggests that the plant extract acted as reductant and converted the silver nitrate into AgNPs.

CCl_4 is a well-known hepatotoxin used in animal models to test liver injury. The major reasons behind CCl_4 -induced hepatopathies are lipid peroxidation, decreased antioxidant enzyme activity, and the generation of free radicals.^{37–39} Hepatotoxins give rise to lipid peroxides by attacking the

fatty acids of membranes, which can cause a loss in functional integrity of hepatic mitochondria, leading to liver damage.⁴⁰ Reducing the generation of free radicals or creating an antioxidant effect are effective ways to treat hepatopathies.⁴¹ Alternative medicines such as herbal medicines rich in antioxidants are therefore the need of the hour due to toxicity issues associated with certain medications.^{42,43} In this context, a large number of plants have been utilized in several countries to treat liver diseases and to enhance liver functions.⁴⁴ The use of plants for treatment of injuries related to the liver has been credited to the involvement of antioxidants as a supplement.³

Among the biochemical analyses performed in this experiment, LDH is a prominent indicator and a diagnostic tool for tissue injury.⁴⁵ LDH levels were elevated in the control group, but were effectively restored to normal in groups treated with test (AgNPs) and standard (silymarin) agents (Table 2). HD treatment of both AgNPs and plant extract were effective in restoring LDH. This indicates the positive effect on injured hepatic cells. Following the analysis of LDH, MDA levels were tested. It is a prominent indicator of lipid peroxidation and oxidative stress which can interact at both genomic and proteomic levels.^{46–48} The MDA level was increased in CCl_4 -treated group, which was restored to near normal levels in treatment groups III–IX (Table 2). LD treatment of plant extract (Group III) and HD treatment of AgNPs (Group VIII) were effective in revival

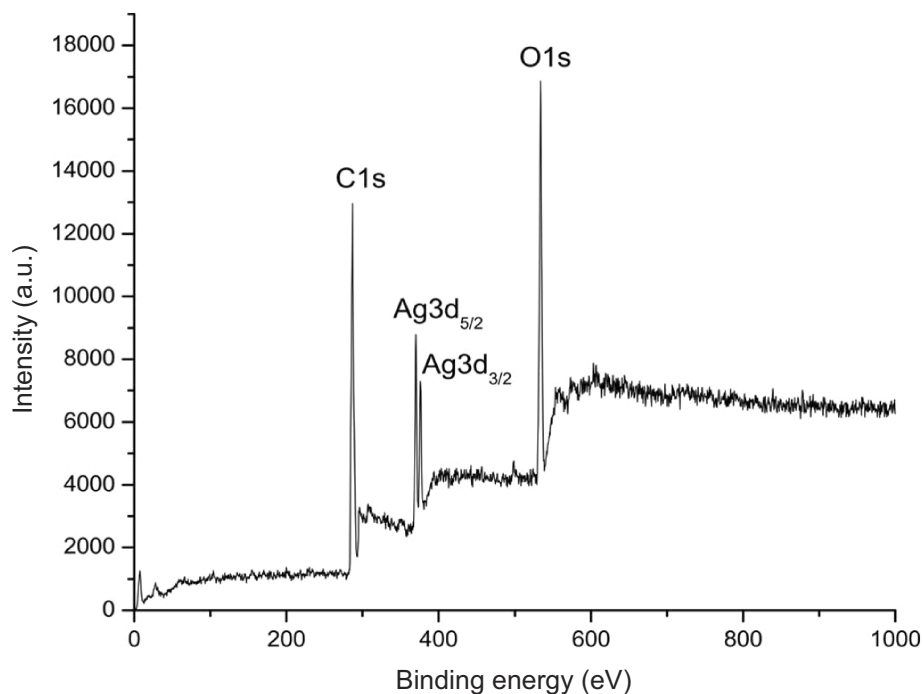


Figure 3 X-ray photoelectron spectroscopy analysis of synthesized silver nanoparticles.

compared to the other treatment groups. These results indicate that the AgNPs had a positive effect on the liver with regard to lipid peroxides. Treatment with HD AgNPs was effective in attenuating the CCl₄-induced injuries.³

ROS levels are maintained by the enzymatic (GPx) and non-enzymatic antioxidants (GSH) as they regulate the production and removal of free radicals under normal conditions.⁴⁹ Both these antioxidants can scavenge free radicals such as H₂O₂ and can defend against oxidative stress.⁵⁰ The enzymatic antioxidant of the study (GPx) was depleted in CCl₄-treated groups. This was effectively restored to near normal in HD plant extract-treated and HD AgNPs-treated groups (Table 2). MD plant extract (Group IV) and HD AgNPs (Group VIII) were effective in restoring GSH, the non-enzymatic antioxidant of the study, to near normal levels (Table 2). Biosynthesized AgNPs have been known to possess antioxidant and free radical scavenging properties.^{51,52} This indicates that the AgNPs had increased the antioxidant potential or, by creating a change in enzymatic system, had caused a significant effect on the revival of the antioxidant system. This may be due to the prevention of the release of free radicals or a protective effect toward the generation of such free radicals.

Increase in serum transaminases is an indication of the structural damage to the liver.⁵³ The subcellular structures affected by CCl₄ exposure are the plasma membrane, endoplasmic reticulum, mitochondria, and golgi apparatus.⁵⁴ This toxic effect releases enzymes of cytoplasmic origin that enter the circulation during liver damage.⁵⁵ Hence, both cell membrane permeability and increased enzyme activity contribute to hepatic structural injury.^{56,57} Increase in circulating levels of liver enzymes is a result of leakage of serum enzymes as a consequence of lipid peroxidation.⁵⁸ In our study, there was an increase in level of serum enzymes such as SGOT, SGPT, and ALP, denoting hepatic damage. Reduction of serum transaminases to near normal levels by the treatment with HD AgNPs (Group VIII), indicates a possible regeneration of hepatocytes and a possible healing effect on the hepatic parenchyma.⁵⁹ The overall revival observed among treatment groups III–IX denotes the curative effect of plant extract and AgNPs. Treatment with HD AgNPs (3.25 mg/kg BW, Group VIII) was effective in treatment considering all the biochemical parameters tested (Table 2).

Histopathological sections of livers of normal animals indicated normal hepatic architecture with distinct hepatocytes and sinusoidal spaces. In the CCl₄-intoxicated group, the hepatic architecture was disrupted, indicating an abnormality among hepatic cells. Tissue necrosis and cytoplasmic

Table 2 Biochemical parameters associated with this study

Parameters	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII	Group VIII	Group IX
Cellular metabolites									
LDH (IUdL ⁻¹)	217.74±13.87 ^e	347.74±13.47 ^a	291.72±20.99 ^b	263.33±22.65 ^{cd}	251.79±9.69 ^d	297.55±13.41 ^b	278.34±13.46 ^{bc}	228.23±16.95 ^e	231.22±13.28 ^e
Lipid peroxidation marker									
MDA (nmoles/g)	20.04±4.85 ^{bcd}	28.78±3.63 ^a	19.45±4.44 ^{bcd}	24.93±4.28 ^{ab}	15.35±1.84 ^{de}	16.25±3.08 ^{de}	12.69±4.13 ^e	20.34±3.49 ^{bcd}	21.45±4.17 ^{bc}
Enzymatic antioxidant									
GPx (IU/mg)	18.97±5.27 ^c	14.98±4.05 ^c	19.01±3.51 ^c	28.33±4.42 ^b	17.69±4.14 ^c	35.42±4.89 ^a	19.76±6.36 ^c	18.69±5.94 ^c	19.88±7.55 ^c
Non-enzymatic antioxidant									
GSH (IU/mg)	24.16±6.98 ^b	18.53±2.73 ^b	21.72±5.81 ^b	26.2±5.12 ^{ab}	20±6.53 ^b	32.99±3.42 ^a	20.41±4.37 ^b	23.16±7.50 ^b	25.11±3.98 ^b
Liver enzymes									
SGOT (IU/l)	152.2±9.94 ^{ab}	163.16±18.14 ^a	117.8±33.74 ^{cd}	138.95±17.17 ^{bc}	134.2±8.91 ^{bc}	108±11.67 ^d	99.4±10.64 ^d	132±15.91 ^{bc}	137.3±18.58 ^{bc}
SGPT (IU/l)	85.75±6.03 ^a	91.98±9.45 ^a	88.8±8.82 ^a	74.27±6.70 ^c	54.86±7.51 ^c	39.49±1.57 ^d	33.65±3.35 ^d	82.88±8.87 ^{ab}	83.82±7.26 ^a
ALP (IU/l)	107.41±11.20 ^{cd}	138.71±13.30 ^b	258.79±24.69 ^a	127.78±10.27 ^b	135.49±4.57 ^b	92.09±10.90 ^d	102.88±9.43 ^{cd}	106.93±9.78 ^{cd}	111.12±6.09 ^c

Notes: Results are expressed as mean ± SD (n=6). Each lower case letter above the SD indicates the significant (p<0.05) difference between the groups. Similar letter represents insignificant change. Different letters determine significant changes among the groups.

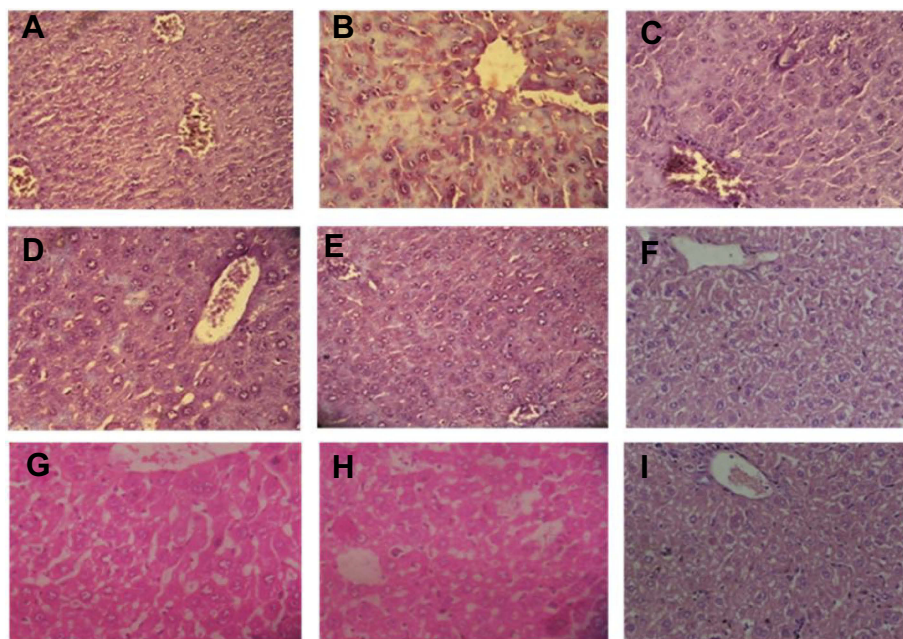


Figure 4 Livers of mice were dissected (after they were euthanized at the end of the experiment), fixed, stained with H&E, and observed at 40× magnification. (A) Normal, (B) control, (C) CCl₄+LD PE, (D) CCl₄+MD PE, (E) CCl₄+HD PE, (F) CCl₄+LD AgNPs, (G) CCl₄+MD AgNPs, (H) CCl₄+HD AgNPs, (I) CCl₄+silymarin. **Abbreviations:** CCl₄, carbon tetrachloride; LD, low dose; PE, plant extract; MD, medium dose; HD, high dose; AgNPs, silver nanoparticles.

vacuolization were observed around the central vein. The cell membranes and central vein were disintegrated. The sinusoidal spaces were dilated. This phenomenon in the intoxicated group was brought back to near normal hepatic architecture in the plant extract- and nanoparticles-treated groups, indicating significant protection of the liver (Figure 4).

The ability of a drug to attenuate injuries or to preserve the normal physiological function of the liver after induction of toxicity is the index of its hepatoprotective effect.⁶⁰ Plant extracts used to synthesize AgNPs can lead to synergistic effects, including an antioxidant effect.⁶¹ Results obtained, considering the biochemical and histological parameters, signify that AgNPs were effective in protection against CCl₄-intoxication. Failure in antioxidant system can lead to an increase in lipid peroxidation and loss of normal cellular functioning in the liver.²⁹ Thus, the antioxidant and free radical scavenging effects of AgNPs might play a crucial role in the revival of the biochemical and histological parameters to near normal in the treatment groups.⁶² This makes AgNPs a valuable candidate for several therapeutic purposes, including protective effects on the liver.

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Disclosure

The authors report no conflicts of interest in this work.

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