Green Synthesis of Near-Infrared Copper-Doped Carbon Dots from *Alcea* for Cancer Photothermal Therapy

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**ABSTRACT:** Heteroatom-doped carbon dots (CDs) with optical absorbance in the near-infrared (NIR) region can provide an opportunity for selective cancer photothermal therapy (PTT). Here, an eco-friendly, simple, cost-efficient, and one-step hydrothermal method was developed to synthesize copper-doped CDs (Cu-doped CDs). The *Alcea* extract as the carbon source was combined with CuSO$_4$ as the dopant. Microscopic and spectroscopic analyses showed that spherical and monodisperse Cu-doped CDs (Cu-dCDs) with sizes below 10 nm have bright fluorescence with photoluminescence quantum yields of 11.1%. Cu-dCDs exhibited an excellent single absorbance peak at 800 nm and strong emission at 460 nm when excited at 370 nm. In vitro low cytotoxicity and the Cu-dCD-mediated cell PTT with the photothermal conversion efficiency (39.3%) show that cell internalization of Cu-doped CDs under an 800 nm NIR laser can induce cell thermal death.

**INTRODUCTION**

Despite the use of various cancer treatment methodologies such as surgery, radiotherapy, and chemotherapy, cancer recurrence is a serious public health problem with approximately 10 million death each year in the world, 1 thus using complementary methods is necessary to prevent the recurrence of cancer. 2

Photothermal therapy (PTT), which induces high heat energy via a focused near-infrared (NIR) laser beam to the tumor, with minimally invasive, no drug resistance, low toxicity, and minimum side effects, is a promising tumor therapy modality. 3−5 In NIR (>700 nm), the biological components (e.g., hemoglobin) have minimum absorbance and allow phototherapy methods to work in a pure window. 6 PTT efficacy can increase by inorganic and organic agents based on light absorbers via generating heat, especially nanoparticles of gold, Pd, Cu, iron oxide, and carbon nanotubes. 7−8 The most often used photothermal agent is Au nanoparticles; however, they can be limited because of the complex synthesis, the optical sensitivity dependent on environmental conditions, clearance mediating to size by the reticuloendothelial system, changing in NIR absorption based on localized surface plasmon resonance by the surrounding medium’s dielectric constant, and shifting in the absorption peak based on the shape. 9 A new alternative photothermal agent can be Cu nanomaterials due to a more cost-efficient synthesis method with a small size than gold nanoparticles. 10 Cu nanoparticles (e.g., CuS) with d–d transitions of Cu$^{2+}$ ion and the absorption wavelength (900 nm) are not affected by changes in the surrounding medium’s dielectric constant, size or shape of nanoparticle, and are ideal for in vivo applications. 11−13 Carbon dots (CDs) (zero-dimensional carbon-based nanomaterials) with a size of below 10 nanometers have unique properties like low toxicity, water-solubility, tunable fluorescence spectrum, high photostability, cell membrane permeability, biocompatibility, and surface functionalization. 14−16 CDs have been used in a diversity of applications such as bioimaging, drug delivery, light-emitting devices, and phototheranostics. 17−22 CDs can be made in mass production from organic and inorganic substances using top-
down and bottom-up synthetic techniques; however, to control the synthesis and the fluorescent (FL) properties of CDs, the bottom-up technique is accepted by more researchers.

Recently, cross-linking or doping CD with heteroatoms (e.g., nitrogen, sulfur, selenium, copper, iron, and metal compounds) is considered as a multifunctional photo diagnostic and therapeutic agent for biological applications. Some reported studies indicated that these nanoparticles have capacities for photodynamic therapy (PDT), PTT, gene, and chemodynamic therapy, and theranostic systems or can boost these therapy methods’ efficiency. Recently, metal-doped CDs, a nanohybrid system, were combined with liposome as a tetramodal imaging agent, regarding gene delivery enhancement and photothermal-chemodynamic cancer synergistic theranostics. The sulfur and nitrogen codoped NIR CDs possessed worthy PTT properties in mouse models with the conversion efficiency of 59%, which accumulated in tumor tissue via passive targeting. Also, gadolinium-doped CDs (Gd@CDs) containing doxorubicin hydrochloride (Dox) as a theranostic system showed effective MRI-guided photothermal chemotherapy.

In addition, theranostic properties of nitrogen-doped mesoporous carbon hollow spheres (NCQD-HCS) have been described with internalization in human oral cancer cells (FaDu) and generation of a significant thermal ablation effect when exposed to the 980 nm NIR laser.

Novel copper-doped CDs with a high quantum yield, high solubility, strong fluorescence, and minimal cytotoxicity from copper complex and polyacrylic acid were employed for fluorescent imaging in both the HeLa (human cervical cancer) cell line and the SH-SYSY (human neuroblastoma cells) multicellular spheroids (3D MCs). Sulfur-doped CDs mixed with copper ions reached a copper/CD cross-linked nanosheets (CuCD NSs), showing photothermal conversion efficiency with noble photothermal stability.

This study aimed to prepare Cu-doped CDs (Cu-CDs) using Alcea leaf extracts as the organic precursor and CuSO₄ as the dopant via a one-step hydrothermal method to achieve noble photothermal compound with the heat-generating capacity under exposure of an 800 nm NIR laser for developing low-cost thermal cancer therapeutics.

![Figure 1. Characterization of Cu-dCDs.](http://pubs.acs.org/journal/acsodf)

**Figure 1.** Characterization of Cu-dCDs. DLS shows that (a) particle size is monodisperse and around 3 nm. (b) Zeta potential of the nanoparticle is negative and around −17.8 mV. (c) TEM and (d) SEM images of synthesized Cu-dCDs show spherical morphology with good dispersion and homogeneous particle.

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**RESULTS AND DISCUSSION**

**Synthesis and Characterization of Cu-dCDs.** An eco-friendly, simple, cost-efficient, and one-step hydrothermal method was developed to synthesize copper-doped CDs (Cu-doped CDs). The Cu-dCDs were synthesized by CuSO₄ and Alcea leaf extract solution precursors via the hydrothermal method. As shown in Figure 1, the dynamic light scattering (DLS) shows that Cu-dCDs are monodispersed with hydrodynamic particle size around 3 nm and zeta potential around −34573. 34574

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−17.8 mV (Figure 1a,b). The TEM and SEM images showed the spherical Cu-dCDs were successfully synthesized with particle size around 3 nm (Figure 1c,d).

Fourier transform infrared (FT-IR) spectra were used for the surface composition of CDs and Cu-dCDs (Figure 2a). The strong absorption bands at 3386 and 2932 cm$^{-1}$ can be attributed to the stretching vibrations of O−H in carboxylic, N−H in R−NH$_2$, and C−H in R−CH$_2$−CH$_3$, respectively. 1630, 1403, and 1074 cm$^{-1}$ associated with the C=O or C=S, CU−OH (according to the previous studies), and C−O vibrations, respectively. The peak at 617 is associated with N−Mg vibration, which realizes the presence of Mg in the chlorophyll structure of all plants. In compression with the FT-IR of CDs (Figure 2a), the peaks around the 3386, 2932, and 600 cm$^{-1}$ that associated with O−H, C−H or N−H, and N−Mg, respectively, are the same with FT-IR of Cu-dCDs, so these groups did not change after Cu doping. In CDs, the other peaks around the 1570, 1458 cm$^{-1}$, and 1211 to 1058 cm$^{-1}$ can be attributed to the stretching vibrations of C=O, C−H, and C−O in carboxylic that matched with the previous FT-IR of CDs. However after the doping process, the peaks related to C=O, C−H, and C−O in the CDs shifted and changed, which shows that the doping process was successful. According to the previous study, copper is mainly coordinated with the −COOH groups. Therefore, shifting and the intensity changes of the C=O (1570 cm$^{-1}$) and the C−O (1074 cm$^{-1}$) in CDs were due to the involvement of electrons on oxygen. Cu and probably SO$_4$ also have doped in

**Figure 2.** Characterization of Cu-dCDs. (a) FT-IR spectrum of CDs and Cu-dCDs. (b) EDS spectra for Cu-dCDs. (c) XRD diffraction pattern of Cu-dCDs. (d) Elemental mapping images of Cu-CDs for the presence and distribution of C, O, N, Cu, and S elements, which are spread in almost all parts.
Optical Properties of Cu-dCDs. UV−vis absorption and PL emission spectroscopy were used to investigate the optical properties of Cu-dCDs. The absorbance peaks of Alcea leaf extracts (444 nm and 670 nm) decreased, whereas the peak in 398 nm slightly increased in CDs, showing the absorbance to 800 nm, showing the Cu doping into the CDs (Figure 3a). The observable peaks in the range of 240−300 nm belong to π→π* transition of C=C or C=C, 350−390 nm to n→π* transition of C=O or C=S, and 400−600 nm to n→π* transition of aromatic sp2 domains with the decline in the CDs and Cu-dCDs. Thus, the optical properties of the CDs and Cu-dCDs are related to particle size, modification, and heteroatom doping, allowing them to be used in more applications.37,38 According to the study reported by Williams et al.39 and the excitation and emission of Cu-dCD nanoparticles, the quantum yield was calculated at 11.1%.

The PL spectra show that maximum excitation is about 398 nm in CDs and about 370 nm in Cu-dCDs (Figure 3b−d). Meanwhile, the highest emission for CDs is at 490 nm (in the range of visible blue light), while in Cu-dCDs shifts to approximately 460 nm. For this reason, when we observe both nanoparticles under the gel doc (in 380 nm), the amount of blue emission of Cu-dCDs is less than CDs (Figure 3e). When the excitation wavelength is increased to 400 nm or 430 nm, the emission peak is red-shifted to about 520 nm. The excitation-dependent emission performance of CDs is associated with the distribution of surface states of nanoparticles with different energy levels. It can be associated with carbon components of organic materials in the synthesis precursor of CDs.40,41 In the production of multi-color luminous CDs, the sp2-conjugated domain, degree of graphitization of CDs, the contents of surface functional groups (e.g., C=O), and being doped with external materials have synergistic effects.8,42,43 Thus, doping CDs with Cu leads to the PL of CDs shifting blue. Under lightroom conditions, after hydrothermal synthesis, the color of the Alceae extract turned yellow, then doping with copper was green with good stability. Under gel doc, the emission of the Alceae extract is red due to the presence of chlorophyll, while CDs and Cu-dCDs have emissions in the blue range and light green, respectively (Figure 3e).

Phoptothermal Properties of Cu-dCDs. The phototherapy efficacies of Cu-dCD concentrations (10, 20, 40, and 80 μg mL−1) were evaluated under an 808 nm laser (1.4 W cm−2) (Figure 4a). Cu-dCDs in the exposure of four cycles of the laser showed high reusability (Figure 4b). The temperature profile of Cu-dCD (80 ppm) solution after the photothermal
conversion efficiency of Cu-dCDs was about 39.3% (Figure 4c).

Moreover, the photothermal conversion efficiency of Cu-dCDs compared with bovine serum albumin (BSA)-coated gold nanorods (GNR@BSA, aspect ratio ∼ 6 nm) (Figure 5). These results indicate that Cu-dCDs have a stronger photothermal conversion efficiency (about 8%) than GNR@BSA with a photothermal conversion efficiency of about 31.6%. It shows that Cu-dCDs can produce heat and can act as a good photothermal agent.

**In Vitro PTT.** The cellular uptake of Cu-dCDs was investigated by atomic absorption (AA) of Cu after 12 h incubation of 4T1 cells with Cu-dCDs, and the cellular uptake was about 71%. This cellular uptake without any coating or ligand can be due to the small size of CDs. Due to the good fluorescence emission of the synthesized nanoparticles (Figure 3d) under the UV lamp, the cells were observed under a fluorescent microscope after incubation with the Cu-dCDs that also show their suitable cell internalization (Figure 6b).

The cytotoxicity of Cu-dCDs was evaluated on 4T1 cells by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) proliferation assay method. Cells were treated with different concentrations of Cu-dCDs (20, 40, 80, and 160 ppm) for 12 h. The cell viability rate was over 80% below 80 ppm (Figure 6a).

To investigate the photothermal ablation of Cu-dCDs, 4T1 cells were incubated with different concentrations of Cu-dCDs and treated with laser irradiation (808 nm, 1 W cm⁻²) for 8 min. Without irradiation, cells have shown more than 85% viability below the 80 ppm concentration, while the cell viability significantly decreased to 37% when treated with an 808 nm laser. The concentration of 40 ppm showed a moderate increase in temperature (about 50 °C) that above 40 ppm significantly increased to over 65 °C. Moreover, it is found that the cell viability is decreased to 15% at 80 and 160 ppm. It is demonstrated that PTT in high temperatures (over 50 °C) generally causes necrosis and below 50 usually causes cell apoptosis. Typically, the temperature range of less than 50 °C produces lower cell necrosis and undesirable immunological reactions, and protecting the surrounding environment is suitable for PTT applications. Thus, 40 ppm concentration of Cu-dCDs was selected for PTT.

Apoptosis have been indicated by DNA damages such as morphological alterations, chromatin condensation, and nuclear fragmentation. The 4',6-diamidino-2-phenylindole (DAPI) staining showed that the control cells were less blue fluorescent within their nuclei and were DAPI-negative (Figure 6c). However, cells incubated with Cu-dCDs (40 ppm) under exposure to an 808 nm laser emitted higher intensity fluorescence. Furthermore, the hallmarks of apoptosis such as the highly condensed chromatin and also nuclear fragmentation were visible.

Annexin V/PI staining analysis (Figure 6d) was performed after 12 h for studying the cell death potential of Cu-dCDs on 4T1 cells. Cells were incubated with Cu-dCDs (40 ppm) and exposed to an 808 nm laser for 8 min, showing an increase in apoptotic population (65.6%) than 12.6% necrosis in 4T1 cells.

**CONCLUSIONS**

The Cu-dopped CDs, as a new type of the PTT agent, were synthesized by a one-step hydrothermal method using *Alcea* leaf extracts and CuSO₄ precursors. The Cu-dCD spheres have a reasonably excellent photothermal conversion of 39.3% and fluorescence quantum yield of 11.1%. Cu-dCD nanosphere-based heat generating shows a good thermal ablation effect on 4T1 cells when exposed to an 808 NIR laser. Cu-dCD-based PTT comparison to the GNR PTT agents shows an easy synthesis and cost-effective method, short synthesis process time, a single absorbance peak at 800 nm, size below 10 nm with great stability, and high cellular uptake. Therefore, the green synthesis of CDs and doping with copper (Cu-dCDs) and other metal elements can be promising for their applications in imaging-guided PTT and cancer therapy in the future.

**METHODS**

**Materials.** Roswell Park Memorial Institute (RPMI) 1640 medium, trypsin—EDTA, penicillin, streptomycin, DAPI, and fetal bovine serum (FBS) were purchased from Gibco BRL.
Life Technologies. The dialysis bags (MWCO = 1 kDa) were purchased from Sigma-Aldrich Company. Methotrexate and MTT and Annexin V/PI were purchased from Sigma-Aldrich Company (St. Louis, MO). Kupfer(II)-sulfate-pentahydrate (CuSO₄·5 H₂O) was purchased from Merck Company.

Methods. Synthesis of Cu-dCDs. The Cu-dCDs were synthesized by the hydrothermal method. Briefly, Alcea leaves were washed to remove the contaminants, cut into small pieces, and powdered by an electric grain grinder. Then, 5 g of powder was mixed in 50 mL of deionized water and 50 mL of the

Figure 5. Size, morphology, UV-vis absorbance, and photothermal properties of GNR@BSA. (a) UV-vis absorption spectrum of GNR@BSA (70 ppm). (b) Corresponding DLS and size distribution of GNR@BSA (100 × 6 nm). (c) TEM and (d) SEM images of the synthesized GNR@BSA, and in SEM image, the BSA coating is more recognizable. (e) Temperature profile of GNR@BSA (70 ppm) solution after laser irradiation (808 nm, 1 W cm⁻²) for 600 s, then the laser was shut off (followed by natural cooling). Inset: the negative natural logarithm of the driving force temperature vs the linear matching of time from the cooling period, and the sample time constant for heat transfer (τₛ) of the GNR@BSA was calculated to be 188.9 s.
alcohol. The mixed solution was sonicated (30 min) and filtered. 6 mg of CuSO₄·5H₂O was added to the solution and transferred into the reactor and heated in an oven for 2 h at 140 °C. The product was filtered, centrifuged (9000 rpm for 15 min), and dialyzed to remove the precipitate. The sample was vacuum-dried to obtain Cu-dCD powder. The CDs were also synthesized by the same method without adding CuSO₄·5H₂O. The GNR@BSA nanoparticles were synthesized by the seedless method that has already been published.

**Characterization of Cu-dCDs.** Cu-dCD morphology, size, and zeta potential were determined by transmission electron microscopy (Hitachi 700, Hitachi High Technologies America, Inc., Pleasanton, CA, Twinsburg, OH, USA) and DLS (Zetasizer Nano ZS90, Malvern Instruments, Malvern, UK). The crystallinity of CDs was examined by XRD on a Rigaku D/max 2500 system with Cu Kα radiation. Fluorescence emission spectra were recorded on a NOVA fiber-coupled spectrometer. The absorption behavior of the Cu-dCDs was recorded using the UV–vis double beam PC 1650 UV–Vis (SHIMADZU, Kyoto, Japan) spectrometer. Structure and composition of Cu-dCDs were observed by FT-IR spectroscopy (TENSOR27–Brucker Spectrometer). The morphology and surface elemental analysis of Cu-dCDs were studied using FESEM and EDS (MIRA3TESCAN-XMU).

The quantum yields were measured using the relative approach reported by Williams et al.\textsuperscript{51}

\[
Q_x = Q_a \frac{F_x A_x \eta_x^2}{F_a A_a \eta_a^2}
\]

where \(Q\) is the quantum yield, \(F\) is the integral of the fluorescence emission scan, \(A\) is the absorbance, \(\eta\) is the index of refraction of the solvent, \(x\) indicates the type of sample to be analyzed, and \(s\) denotes the reference material. Quinine sulfate

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**Figure 6.** MTT assay, DAPI staining, and flow cytometry of 4T1: (a) 4T1 cells viability in the Cu-dCD concentrations (20, 40, 80, and 160 ppm) for 12 h. (b) Cellular uptake and cell internalization of Cu-dCDs under the fluorescence microscope. (c) Cells in the control group were normal and were stained with a less blue fluorescence in nuclear (DAPI-negative). In addition, in cells incubated with Cu-dCDs (40 ppm), no abnormalities were identified. However, in the cells incubated with Cu-dCDs (40 ppm) and exposed to an 808 nm laser for 8 min, nuclear fragmentation is visible, and cells displayed high intensity of blue fluorescence, resulting in highly condensed chromatin. (d) Annexin V/PI staining was conducted by flow cytometry for the quantitative study of early apoptotic, late apoptotic, and necrotic 4T1 cells after 12 h treatments for the control group, Cu-dCDs, and Cu-dCDs exposed to an 808 nm laser for 8 min.
(with a fluorescence quantum yield of 0.54) was selected as the standard fluorescent agent because of the similar excitation and emission wavelengths of the produced Cu-dCDs. The samples were diluted to eliminate errors from re-absorption and internal reflection.

**Photothermal Performance.** Photothermal performance of Cu-dCDs and GNR@BSA were taken in a laser (PSU-III-LED, Changchun New Institute, China model). Cu-dCDs and GNR@BSA aqueous solutions (0.3 mL) irradiated with an 808 nm NIR laser (1 W cm\(^{-2}\)). After reaching steady-state temperature (480 s), it was cooled to room temperature without NIR laser (followed by natural cooling). The temperature changes of the solutions were measured using a digital thermometer (LCD K-Type Digital Thermometer, Shenzhen, China). The photothermal conversion efficiency (\(\eta\)) was measured by the previously described method\(^{1,3}\) and the following equation

\[
\eta = \frac{h s (T_{\text{max}} - T_{\text{surr}}) - Q_{\text{ds}}}{I (1 - 10^{-A_{\text{surr}}})}
\]

where \(h\) is the heat-transfer coefficient, \(S\) is the surface area of the container, \(T_{\text{max}}\) is the equilibrium temperature (\(T_{\text{max}} = 71.3\) °C), \(T_{\text{surr}}\) is the room temperature (\(T_{\text{surr}} = 21\) °C), and \(Q_{\text{ds}}\) is the heat dissipation due to the light absorbed by the quartz sample cell, and it was measured independently to be 28.2 mW using a quartz cuvette cell containing pure water. I is the laser power (1 W cm\(^{-2}\)), and \(A_{\text{surr}}\) is the absorbance of the Cu-dCDs solution at 808 nm (1.892). The value of \(h s\) was derived according to the following equation

\[
h s = \frac{m C_p}{\tau_i}
\]

where \(m\) is the solution mass (0.3 g), \(C_p\) represents the heat capacity [4.2 J/(g·°C)] of water, and \(\tau_i\) in the sample system time constant is measured using the following equation

\[
\tau_i = \frac{t}{-\ln \theta L}
\]

\[
\theta = \frac{T_{\text{th}} - T_{\text{surr}}}{(T_{\text{max}} - T_{\text{surr}})}
\]

\(T_{\text{th}}\) indicates the solution temperature. \(\tau_i\) is derived through fitting the linear time data from the cooling period (after \(T_{\text{max}}\)) versus negative natural logarithm of driving force temperature (\(-\ln \theta\)), and it was 149.6 (s) (Figure 4c). For GNR@BSA, the photothermal conversion efficiency (\(\eta\)) has been measured, as shown in Figure 5d.

**Cytotoxicity Assays and Cellular Uptake.** The 4T1 cells were cultured in the complete medium [RPMI 1640 medium; 10% (v/v) fetal bovine serum and 1% (v/v) penicillin-streptomycin] in 25 cm\(^2\) culture flasks at 37 °C, 5% CO\(_2\) until at least 70% confluency. Then, the cytotoxicity of Cu-dCDs was evaluated on 4T1 cells using standard MTT assay. 4T1 cells were seeded at 2 × 10\(^4\) cells/100 \(\mu\)L/well in 96-well plates and incubated overnight at 37 °C, with 5% CO\(_2\). Then, cells were treated with various concentrations of Cu-dCDs (0.0, 20, 40, 80, and 160 ppm). The MTT solution was added to cells and incubated for 4 h at 37 °C. After removing MTT, 200 \(\mu\)L of DMSO (Sigma-Aldrich) was added to the cells, and the absorbance was measured at 570 nm.

The DAPI staining was analyzed, as described by Rashmi et al. (2003). 3 × 10\(^6\) cells incubated on six-well plates then exposed to the Cu-dCDs (40 ppm) for 12 h and were gently scraped and harvested by centrifugation. The cells were fixed with 3% paraformaldehyde, permeabilized with 0.2% Triton X-100, and incubated with DAPI. Finally, the condensed fragmented chromatin was determined under a fluorescent microscope (Olympus, Japan). The CU-dCD cell internalization was determined in the same way as described above.

An in vitro cellular uptake of Cu-dCDs was determined using the AA spectrometer (NovaAA400, Analytik Jena). Cells were incubated in the presence of 40 ppm for 12 h. Then, the cells were detached, centrifuged, and washed twice with phosphate-buffered saline (PBS). The cell pellets were resuspended in 1.0 mL of twice-distilled water and lysed. The AA was used to assess the Cu.

**PTT Performance In Vitro.** 4T1 cells were cultured in a 96-well microplate. Then, Cu-dCDs with different concentrations (0, 20, 40, 80, and 160 ppm) were added and incubated for 12 h. Then, the cells were exposed to an 808 nm laser (1 W) for 10 min, and 12 h later, the cell viability was measured by the MTT method, as described above.

**Flow Cytometry Analysis.** The 4T1 cells were subjected to the FACS flow cytometer. Cells were incubated on six-well plates with Cu-dCDs (40 ppm) at 37 °C in a CO\(_2\) incubator for 12 h. Then, each well was exposed to the laser 808 nm. The cells were trypsinized, centrifuged, and washed with cold PBS. The cell pellets were suspended in 100 \(\mu\)L of annexin binding buffer and incubated under the culture condition for 15 min. Then, 5 \(\mu\)L of annexin V and 5 \(\mu\)L of PI were added and incubated for 15 min at room temperature. The percentage of early and late apoptosis was investigated by a FACSC flow cytometer (Becton Dickinson).

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**Notes**

The authors declare no competing financial interest.

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