The role of curcumin in streptozotocin-induced hepatic damage and the trans-differentiation of hepatic stellate cells

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A B S T R A C T

Diabetic patients frequently suffer from non-alcoholic steatohepatitis. The current study aimed to investigate the role of curcumin and the response of hepatic stellate cells in streptozotocin (STZ)-induced hepatic damage. Sixty male rats were divided into three groups. The normal control injected with a citrate buffer vehicle and the diabetic control group which was injected intraperitoneally (IP) with a single-dose of streptozotocin (30 mg/kg body weight) and a diabetic group was treated with an oral dose of curcumin at 80 mg/kg body weight daily for 60 days. Curcumin effectively counteracts oxidative stress-mediated hepatic damage and improves biochemical parameters. Alpha-smooth muscle actin (α-SMA) was significantly reduced, and insulin antibodies showed strong positive immunoreactivity with curcumin administration. These results optimistically demonstrate the potential use of curcumin, which is attributed to its antiradical/antioxidant activities and its potential β-cell regenerative properties. Also, it has the capability to encourage the trans-differentiation of hepatic stellate cells into insulin-producing cells for a period of time. In addition, as it is an anti-inflammatory mediator that inhibits hepatic stellate cell activation and the transition to myofibroblast-like cells, this suggests the possibility of considering curcumin’s novel therapeutic effects in reducing hepatic dysfunction in diabetic patients.

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1. Introduction

Diabetic patients frequently suffer from a hepatic impairment recognized as non-alcoholic steatohepatitis which is associated with severe complications such as deposition of glycogen, steatosis, cirrhosis, fibrosis and occasionally hepatic cancer (Bugianesi et al., 2007).

Streptozotocin (STZ) is an N-nitroso-N-methyleurea derivative of 2-deoxy-D-glucose which is a diabetogenic agent that damages the islet β-cells in the pancreas selectively to produce insulin-dependent diabetes mellitus (IDDM) (Yang et al., 2010).

Alpha-smooth muscle actin (α-SMA) is an indicator for the recognition of myofibroblast-like cells plus hepatic stellate cells (HSCs) which are also known as Ito cells (Clement et al., 2010). In diabetes, the glucose accessibility is increased and this leads to an accelerated formation of advanced glycation end products (AGEs). AGEs interact with the receptor for AGEs (RAGEs) and consequently increase oxidative stress and cellular growth. This leads to an increased proliferation of HSCs, which is noticed during hepatic fibrogenesis that is accompanied by the up-regulation of RAGES.

Oxidative stress plays a crucial role in the chronic complications of the diabetic liver, where it is associated with an overproduction of oxygen free radicals and lipid peroxidation (Saravanan and Ponnurugan, 2011).

The use of herbal medicine in defending against STZ-induced liver damage looks promising. Curcumin has been gaining attention because of its health benefits, such as its anti-inflammatory, antioxidant, and immune modulatory effects. Curcumin is the chief curcuminoi d of Curcuma Longa, which is a member of the Zingiberaceae family (Kumar et al., 2015).

The polyphenol curcumin improves diabetes-induced dysfunction by decreasing the level of glucose, inhibiting protein-kinase C, and lowering superoxide production (Rungeesantisavvan et al., 2010). It reverses insulin resistance, hyperglycemia, and hyperlipidemia by inhibiting the pro-inflammatory transcription factors, signal transducers and stimulating anti-inflammatory signaling pathways (Tang and Chen, 2010).

This study aimed to investigate the probable influence of hepatic stellate cells in the protective capability of curcumin in STZ-induced liver damage. It was also intended to provide an important support in understanding the mechanism of curcumin treatment for hepatic dysfunction.

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2. Materials and methods

2.1. Ethical approval

This study was conducted after receiving the approval of the Medical Research Ethics Committee, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia.

2.2. Chemicals and Reagents

Streptozotocin (STZ) and curcumin were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) kits were purchased from Randox Laboratories Ltd. (Crumlin, County Antrim, UK). Serum albumin, total protein, and total bilirubin were determined by spectrophotometer using the corresponding colorimetric kits supplied by Bio Diagnostic Company (Cairo, Egypt).

2.3. Animals

Sixty male adult albino Wistar rats, weighing 190 ± 20 g, that were housed were obtained from the animal house. Animals were housed in a (24 °C ± 3 °C) temperature-controlled room with 40–70% humidity and 12/12 h light/dark cycle. Rats were fed a standard diet and tap water ad libitum throughout the experiment. The experimental procedures were performed in accordance with the international guidelines for the care and the use of animals in a laboratory.

2.4. Induction of diabetes

Fasted rats (12 h) received a single intraperitoneal (IP) injection of freshly prepared STZ (50 mg/kg body weight) dissolved in 0.1 M citrate buffer (pH 4.5). STZ-injected animals were given a 5% glucose solution for 24 h to overcome drug-induced hypoglycemia. On the third day after STZ injection, glucose levels were estimated by obtaining blood samples from the cut tip of the tail using Diagnostic Accu-Chek test strips (Roche Diagnostics, Mannheim, Germany). Blood glucose levels of 250 mg/dl or more were considered diabetic.

2.5. Experimental design

The animals were distributed into 3 groups (20/group). Group 1 (normal control) was injected IP with a citrate buffer vehicle. Group 2 (diabetic control) received a single IP injection of STZ (50 mg/kg body weight). Group 3 received a single IP injection of STZ (50 mg/kg body weight) and, on the third day after the STZ injection, curcumin was given orally with a dosage of 80 mg/kg body weight and continued daily for 60 days (Zhang et al., 2013). At the end of the experiment, blood samples were collected from the retro-orbital sinus in heparinized capillary tubes for serum analysis. Animals from all groups were sacrificed, and the livers were processed for histological studies.

2.6. Plasma glucose estimation

Plasma glucose was measured using an enzymatic colorimetric method with commercially available kits (Randox Laboratories, Ltd., Antrim, UK).

2.7. Plasma insulin estimation

Plasma insulin was determined using an insulin enzyme-linked immunosorbent assay (ELISA) kit (code no. AKRIN-010T; Shibayagi Co., Ltd., Gunma, Japan).

2.8. Serum parameters

ALT, AST, and ALP, which were increased following hepatocyte injury, were assessed according to the protocol detailed in the manuals of the diagnostic kits (Randox Laboratories Ltd., Crumlin, County Antrim, UK). Serum albumin, total protein, and total bilirubin were determined by spectrophotometer using the corresponding colorimetric kits supplied by Bio Diagnostic Company (Cairo, Egypt) (Lee et al., 2012).

2.9. Histological examination

Livers were washed with a phosphate buffer solution and then fixed in 10% neutral buffered formalin. Tissues were dehydrated through a graded series of alcohol, cleared in xylene, and embedded in paraffin wax. Tissues were then cut into sections of 3–5 μm in thickness using a microtome and stained with hematoxylin and eosin (H&E) for histopathological evaluation and with periodic Acid-Schiff (PAS) for observation of glycogen. For each specimen, at least three to five slides were examined using an Olympus BX53 microscope equipped with a DP73 camera (Olympus, Tokyo, Japan).

2.10. Histopathological evaluation

The sections were analyzed for hydropic swelling, parenchymatous degeneration, microvesicular vacuole, macrovesicular vacuole, focal necrosis, inflammatory infiltrations, fibrosis, and sinusoids hyperemia. At the end of the analyses, the findings were presented in a table which showed the degree of degeneration (Güven et al., 2006). Score levels of 0, +1, +2, +3 were equivalent to no, mild, moderate, and severe, respectively. The scores represented values obtained from the tissue sections of six animals from each group with five fields/section (Mustafa et al., 2015).

2.11. Immunohistochemical examination

The standard peroxidase immunohistochemistry technique was applied to slides of paraffin-embedded tissue. Sections were de-waxed in xylene, rehydrated, and pretreated with 3% of hydrogen peroxide solution to block endogenous peroxidase activity. Microwave-assisted antigen retrieval was performed for 20 min. Slides were then incubated overnight at 4 °C with the primary antibody against α-SMA (a mouse monoclonal antibody [Dako, Carpinteria, California, USA] with a dilution of 1:50; cellular site was cytoplasmic) as a marker of activated HSCs with varying degrees of intensity in smooth muscles and myofibroblasts. They were similarly incubated with the primary antibody against insulin (a mouse monoclonal antibody [Dako, Carpinteria, California, USA] with a dilution of 1:100; cellular site was cytoplasmic). The sections were incubated with biotinylated IgG and then with streptavidin-peroxidase conjugate (Zymed Corp, San Francisco, CA, USA). Sections were then washed with phosphate-buffered saline (PBS) and incubated with 3, 3′-diaminobenzidine tetrachloride (DAB) substrate chromogen solution (1 drop of DAB chromogen/1 mL of substrate buffer) for 5 min to detect immunoreactivity. All sections were counter-stained with Mayer’s hematoxylin. Negative control sections were prepared by omitting the primary antibody. Positive control standard laboratory slides were used for all stains to prove the success of the technique. All slides were examined under light microscopy, and the presence of labeled cells was documented. Absence of staining was recognized as a negative result (−), while the presence of brown staining was recognized as positive result (+) (Mustafa et al., 2015).
2.12. Quantitative morphometric measurements

Ten non-overlapping fields for each animal were selected indiscriminately and analyzed. The measurements were done by using Image-Pro Plus v6.0 software (Media Cybernetics, Silver Spring, MD, USA) and the NIH Imagej (v1.50) program (http://rsb.info.nih.gov/ij/) with an Olympus BX53 microscope (Olympus, Tokyo, Japan). The area percentages of α-SMA immunopositive cells and the insulin-positive cells number were evaluated (Hussein et al., 2015).

2.13. Statistical analysis

Quantitative data were expressed as the mean ± SD of different parameters for the treated groups. The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test. The statistical analysis was performed using IBM SPSS version 23. The values were considered significant when p < 0.05.

3. Results

3.1. Effect on liver and body weight

At the end of 60 days, curcumin treated specimens showed a significant increase in the body and liver weight as compared to the diabetic control (Table 1 and Fig. 1).

3.2. Biochemical parameters

The mean glucose level of the diabetic control group was significantly increased while curcumin treatment showed a significant improvement in glucose level (Table 2 and Fig. 2). The mean insulin level of the diabetic control group was significantly decreased while curcumin treatment showed a significant improvement in insulin level (Table 2 and Fig. 2).

Plasma ALT, AST, and ALP levels were elevated in the diabetic control group in comparison to those of the normal control group, while with curcumin treatment all of these parameters are reduced significantly. Furthermore, there was a significant decrease in the albumin and total protein in the diabetic control group, while curcumin treatment showed a significant increase in these parameters compared to those of the diabetic control group. Conversely, the total bilirubin was increased in the diabetic control group and decreased significantly with curcumin treatment (Table 2 and Fig. 3).

3.3. Histopathological findings

The normal control group showed normal cytoarchitecture. The diabetic control group showed parenchymatous degeneration and loss of architecture. With curcumin treatment, hepatic changes were reduced (Table 3).

PAS-stained sections of the normal control group showed PAS-positive red granules in the cytoplasm of the hepatocytes and more granules around the central vein (Fig. 4A), while those of the diabetic control group showed few PAS-positive granules (Fig. 4B). With curcumin treatment, mild PAS-positive granules were observed (Fig. 4C and Table 3).

3.4. Immunohistochemistry-stained sections

In the normal control group, α-SMA staining was observed only in the media of the blood vessels (portal veins, hepatic artery branches, and central veins) (Fig. 5A). While the diabetic control group showed positive α-SMA immunoreactivity in the media of the portal vessels and the perportal area and along the perisinusoidal spaces (Fig. 5B and C). In the curcumin treatment group, mild immunoreactivity was observed (Fig. 5D). The area percentage of α-SMA positive cells was significantly higher in the diabetic control group, while in the curcumin treatment group it was reduced significantly (Table 4 and Fig. 7).

Insulin-immunostained sections for both the normal control (Fig. 6A) and diabetic control (Fig. 6B) groups showed no or minimal insulin immune positive cells. The curcumin treatment group showed many insulin positive immune cells in the portal area around the bile ductule (Fig. 6C). The number of insulin positive cells was significantly higher in the curcumin treated group (Table 4 and Fig. 7).
Fig. 2. Plasma glucose and insulin of different groups. The mean is given in columns, and error bars represent the standard deviation (SD).

Table 2
Biochemical parameters of the different groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control</th>
<th>STZ-diabetic control</th>
<th>STZ-diabetes + curcumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma glucose (mg/dl)</td>
<td>87.14 ± 4.28</td>
<td>280.12 ± 57.24</td>
<td>160.12 ± 63.27</td>
</tr>
<tr>
<td></td>
<td>*P &lt; 0.001</td>
<td>*P &lt; 0.001</td>
<td>*P &lt; 0.001</td>
</tr>
<tr>
<td>Plasma insulin (mIU/L)</td>
<td>5.17 ± 0.73</td>
<td>0.54 ± 0.11</td>
<td>4.06 ± 0.45</td>
</tr>
<tr>
<td></td>
<td>*P &lt; 0.001</td>
<td>*P &lt; 0.001</td>
<td>*P &lt; 0.001</td>
</tr>
<tr>
<td>ALT (U/I)</td>
<td>65.4 ± 1.2</td>
<td>119.6 ± 2.30</td>
<td>76.8 ± 4.50</td>
</tr>
<tr>
<td></td>
<td>*P &lt; 0.001</td>
<td>*P &lt; 0.001</td>
<td>*P &lt; 0.001</td>
</tr>
<tr>
<td>AST (U/I)</td>
<td>139.13 ± 3.82</td>
<td>321.35 ± 2.80</td>
<td>204.68 ± 1.96</td>
</tr>
<tr>
<td></td>
<td>*P &lt; 0.001</td>
<td>*P &lt; 0.001</td>
<td>*P &lt; 0.001</td>
</tr>
<tr>
<td>ALP (U/I)</td>
<td>68.10 ± 2.30</td>
<td>85.2 ± 4.60</td>
<td>79.5 ± 8.10</td>
</tr>
<tr>
<td></td>
<td>*P &lt; 0.001</td>
<td>*P &lt; 0.001</td>
<td>*P &lt; 0.001</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>4.14 ± 0.60</td>
<td>1.3 ± 0.87</td>
<td>3.81 ± 0.40</td>
</tr>
<tr>
<td></td>
<td>*P &lt; 0.001</td>
<td>*P &lt; 0.001</td>
<td>*P &lt; 0.001</td>
</tr>
<tr>
<td>Total protein (g/dl)</td>
<td>5.94 ± 0.89</td>
<td>2.03 ± 0.78</td>
<td>4.62 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>*P &lt; 0.001</td>
<td>*P &lt; 0.001</td>
<td>*P &lt; 0.001</td>
</tr>
<tr>
<td>Total bilirubin (mg/dL)</td>
<td>0.24 ± 0.20</td>
<td>0.78 ± 0.07</td>
<td>0.36 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>*P &lt; 0.001</td>
<td>*P &lt; 0.001</td>
<td>*P &lt; 0.001</td>
</tr>
</tbody>
</table>

Values are means ± SD (n = 20 each group). ANOVA followed by Tukey’s post hoc test.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; mg/dl, milligrams per deciliter; mIU/L, milli-international units per liter; U/I, units per liter; g/dl, grams per deciliter.

4. Discussion

In the present study, the diabetic control group revealed a reduction in body weight, which is attributed to the muscle destruction or catabolism of structural protein and fat (Salahuddin and Jalalpure, 2010). Curcumin administration improves the body weight, and this indicates the effect of curcumin on the degradation of structural protein, control of muscle wasting, and increase of metabolic processes in the body.

The current data showed a significant decrease in the plasma insulin levels, which coincides with other findings (Abdel Aziz et al., 2013). Improved insulin level supports the hypothesis that curcumin causes β-cells neogenesis and protection (Meghana et al., 2007), and this was evidently proven by this study. Furthermore, it is proposed that controlling hyperglycemia encourages further regeneration of β-cells (Guz et al., 2001). This may be attributed to the suggestion that dying β-cells are regenerated continuously (Montanya et al., 2000).

Hepatocellular injury is credited to reactive oxygen species (ROS) and lipid peroxidation that causes direct damage to hepatocytes by disrupting the membranes, protein, and DNA (Asmah Rahmat, 2015). The injured hepatocytes release aldehyde end products, which causes further hepatocellular damage and emergence of fibrosis (Novo and Parola, 2012). Also, lipid peroxidation causes an inflammatory reaction that involves the occurrence of cytokines, mainly tumor necrosis factor (TNF-α), interleukin-1 (IL-1), and consensus interferon (IFN-γ) (Sugano et al., 2006). Meanwhile, curcumin treatment alleviates the hepatocellular injuries.

In the present study, the diabetic control group revealed a congestion of the portal triad with inflammation and notable fibrosis near the central vein. This is attributed to tissue degradations that are caused by depletion of the endogenous antioxidant enzyme stores as superoxide dismutase (SOD) and catalase (CAT) (Chang and Chuang, 2010) and promotion of de novo generation of free radicals (Maritim et al., 2003). Consequently, the higher level of antioxidants that exists in curcumin could boost the quenching of some free radicals inside hepatocytes, which defend the hepatic tissue against oxidative stress damage (Afrin et al., 2015), and this is supported by the current findings.

In liver impairment, hepatic stellate cells (Ito cells) undergo trans-differentiation from lipid storing pericytes to myofibroblast-like cells that lead to an increased collagen and extracellular matrix that is the crucial event in hepatic fibrogenesis (She et al., 2005). Curcumin treatment in the current study caused a reduction of α-SMA positive cells, which is the marker of Ito cell activation and in turn, indicates inhibition of Ito cells and the improvement of hepatic fibrosis (Erenoglu et al., 2011).

Curcumin suppresses the AGES mediated by the stimulation of RAGEs gene expression. This leads to inhibition of the RAGE activated pathways which, in turn, prevents oxidative
stress, inflammation, and hepatic stellate cell activation, a hallmark of non-alcoholic steatohepatitis and hepatic fibrogenesis associated with type 2 diabetes mellitus (Stefanska, 2012). Moreover, AGE-encouraged production of reactive oxygen species and lipid peroxides was attenuated after treatment with curcumin (Stefanska, 2012).

In the current findings, the trans-differentiation of Ito cells into myofibroblast-like cells is attributed to intercellular connections between Ito cells and activated Kupffer cells, damaged hepatocytes, and inflammatory cytokines (Hellerbrand, 2013). Other factors that cause this trans-differentiation include the platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and transforming growth factor β1 (TGF-β1) that are released after activation of Kupffer cells (Nosseir et al., 2012). In hyperglycemic conditions, the diabetic control group in the current study showed minimal insulin immune reactions of hepatic stellate cells. This indicates that diabetes encourages the transition of hepatic stellate cells to insulin-producing cells but it cannot maintain their production. Furthermore, it was found that this trans-differentiation occurs early and lasts for two weeks in the diabetic liver (Kim et al., 2007). This transition is attributed to the fact that both the liver and the pancreas originate from the upper primitive foregut endoderm appendages (Lammert et al., 2003) and the late separation of the pancreas and liver during organogenesis might leave pluripotent cells, which are able to give rise to both pancreatic and hepatic lineage (Meivar-Levy and Ferber, 2003).

In supporting this hypothesis, a previous study has discussed the existence of extra-pancreatic insulin-producing cells in the liver in the hyperglycemic condition. However, the number and production of these insulin-producing cells are not enough to improve the hyperglycemic condition (Kojima et al., 2004). The current results support the hypothesis that the hypoglycemic effect of curcumin is attributed to preserving functional insulin-producing cells. In the curcumin group in the current study, insulin immune positive cells were increased which supports the suggestion that curcumin may maintain insulin production by trans-differentiated hepatic stellate cells for a period after STZ injection.

These results optimistically demonstrate the potential use of curcumin for the treatment of STZ-induced liver damage because of its antiradical/antioxidant activities, potential β-cell regenerative properties, and capability to encourage the trans-differentiation of hepatic stellate cells into insulin-producing cells for a period of time. In addition, as it is an anti-fibrotic mediator that inhibits hepatic stellate cell activation and the transition to

### Table 3

<table>
<thead>
<tr>
<th>Histopathological findings of the liver.</th>
<th>Normal control</th>
<th>STZ-diabetic control</th>
<th>STZ-diabetes + curcumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydropic swelling</td>
<td>0</td>
<td>+2</td>
<td>+1</td>
</tr>
<tr>
<td>Parenchymatous degeneration</td>
<td>0</td>
<td>+2</td>
<td>+1</td>
</tr>
<tr>
<td>Microvesicular vacuole</td>
<td>0</td>
<td>+0.5</td>
<td>+1</td>
</tr>
<tr>
<td>Macrovesicular vacuole</td>
<td>0</td>
<td>+0.5</td>
<td>+1</td>
</tr>
<tr>
<td>Focal necrosis</td>
<td>0</td>
<td>+2</td>
<td>+0.5</td>
</tr>
<tr>
<td>Inflammatory infiltrations</td>
<td>0</td>
<td>+1</td>
<td>+1</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>0</td>
<td>+1</td>
<td>+0.5</td>
</tr>
<tr>
<td>Sinusoids hyperemia</td>
<td>0</td>
<td>+2</td>
<td>+1</td>
</tr>
<tr>
<td>PAS</td>
<td>+3</td>
<td>+1</td>
<td>+2</td>
</tr>
</tbody>
</table>

N = 20; Scale: No (0), mild (+1), moderate (+2), severe (+3).
Fig. 4. (A) Normal control group showed PAS-positive reaction (distribution of liver glycogen) (arrow); (B) diabetic control group showed few PAS-positive reaction (arrow); (C) curcumin treatment group showed mild PAS-positive reaction (arrow) (PAS, scale bar 20 μm).

Fig. 5. (A) Normal control group showed minimal immunostaining (arrow); (B) diabetic control group showed positive immunostaining (activated HSCs) in the media of the portal blood vessels and scattered in the periportal area (arrow); (C) the previous group with positive immunostained cells in the perisinusoidal spaces (arrow); (D) curcumin treatment group showed mild immunostaining (arrow) (α-SMA, scale bar 20 μm).
Table 4
Mean ± SD of the area percentage of α-smooth muscle actin positive cells and the number of insulin-positive cells.

<table>
<thead>
<tr>
<th></th>
<th>Normal control</th>
<th>STZ-diabetic control</th>
<th>STZ-diabetes + curcumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area % of α-SMA</td>
<td>0.43 ± 0.28</td>
<td>10.20 ± 0.62</td>
<td>1.06 ± 0.87</td>
</tr>
<tr>
<td></td>
<td>*P &lt; 0.001</td>
<td>*P &lt; 0.01</td>
<td>*P &lt; 0.001</td>
</tr>
<tr>
<td>Number of insulin positive cells</td>
<td>0.00</td>
<td>0.28 ± 0.01</td>
<td>5.10 ± 3.50</td>
</tr>
<tr>
<td></td>
<td>*P &gt; 0.05</td>
<td>*P &lt; 0.001</td>
<td>*P &lt; 0.001</td>
</tr>
</tbody>
</table>

Values are means ± SD (n = 20 each group). ANOVA followed by Tukey’s post hoc test. *P: compared to normal control. **P: compared to diabetic control.

myofibroblast-like cells, consequently the way is now open to consider curcumin’s novel therapeutic effects in reducing hepatic dysfunction in diabetic patients.

5. Conclusion

The current data suggest that curcumin affects hepatic stellate cell activation, which represents a key event of non-alcoholic steatohepatitis and hepatic fibrogenesis associated with diabetes.

Conflicts of interest
None.

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