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Protective Effect of Kaempferol against Hydrogen Peroxide-Induced Oxidative Damage in HUVECs Cell Line

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Authors' Contribution
LM designed this study. XH and BY performed the experiment and analyzed the data. CZ provided experimental operation support.

Key words
Kaempferol, H_2O_2, human umbilical vein endothelial cells, MTT, Flow cytometry, Western blotting

ABSTRACT

In order to understand the protective impact of kaempferol, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay was applied in hydrogen peroxide-treated human umbilical vein endothelial cells (HUVECs). The hydrogen peroxide-induced apoptosis was gauged using flow cytometry whereas western blotting was applied to detect the expression of key cellular markers in oxidation pathways. The data indicate that a total of 50 µmol/l concentration of kaempferol can profoundly protect the oxidative damage in HUVECs. However, compared to the oxidation-treated cells, the apoptosis rate was significantly decreased in drug-treated cells. Mechanistically, the percentage of G1 phase and S phase cells in vitamin C and drug groups was decreased, and the percentage of G2 phase cells was increased significantly. Intriguingly, the number of CD105/CD62E-marked HUVECs cells was markedly observed. In comparison to the oxide-treated cells, the expression level of p16, p53, p21 and Bax protein was reduced significantly in the drug-treated cells and the expression level of Bcl-2 protein increased significantly. Taken together, kaempferol can inhibit cell apoptosis by protecting the oxidative damage in HUVECs, possibility by reducing p16, p53, p21 and Bax protein expression and increasing the Bcl-2 protein expression levels in the HUVECs cells. These finding underlay some of the key markers associated with the hydrogen peroxide induced oxidative damage and may guide future therapeutics, and pharmaceutical interventions.

INTRODUCTION

Electron transfer is a fundamental change in broad range of biological systems and free radicals are physiological metabolites of human body. Under normal conditions, free radicals are in a dynamic balance of continuous generation and elimination (REF). However, substantial increase in the number of free radicals can lead to damage of the cell structure, cause lipid peroxidation, interfere with the normal metabolic activities of the body, cause disease and can accelerate the process of human aging.

Flavonoids are widespread natural plant phenolic compounds (González-Gallego *et al*., 2014), and their structure contains C6-C3-C6 basic skeleton. The parent nucleus has extensive pharmacological activities (Guan and Liu, 2016; Zhang *et al*., 2017) such as antioxidant, anti-aging, anti-tumor, antibacterial, anti-inflammatory, anti-tumor and regulating vascular permeability among others. These myriads of application in medicine, cosmetics and health propose broad market prospects.

Kaempferol is a well-known flavonoid and is widely found in vegetables, fruits, flowers and Chinese herbal medicine. The kaempferol has multiple clinical application including anti-oxidation and anti-atherosclerosis. Additionally, the effects of quercetin and kaempferol on blood glucose and lipid levels in diabetic mice has been suggested (Zhang, 2013) studied. Lian, 2014 and colleagues have shown that kaempferol can inhibit BaP-induced oxidative damage in HTR8-SVneo cells by increasing the activity of antioxidant enzyme known as SOD. It has also been shown that kaempferol can reduce the production of lipid peroxide MDA and NO by underlining the apoptosis in BaP injury model (Dang *et al*., 2015).

Here, we attempted to study the protective impact of kaempferol in hydrogen peroxide-treated human umbilical vein endothelial cells (HUVECs) to reveal the antioxidant functions of kaempferol and to provide mechanistic insights into the clinical antioxidant application of kaempferol.

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MATERIALS AND METHODS

Cells, reagents and main instruments

M199 medium, fetal bovine serum, EDTA, trypsin, annexin V-FITC/PI apoptosis detection kit, anti-CD105-FITC, anti-CD62E-PE, whole protein extraction kit, RIPA lysate, and SDS-PAGE gel preparation kit were purchased from Sigma. The PVDF membrane (Millipore), β-actin, mouse anti-human p16, mouse anti-human p53, mouse anti-human p21 antibodies were procured from BD. Imaging were acquired using inverted biological microscope from Olympus.

HUVECs cell line culture

Culture of HUVECs were maintained in M199 medium supplemented with 10% fetal bovine serum. Cells were incubated at 37°C constant temperature under 5% CO_2. The cells were sub-cultured once in 2-3 days. All experiments were conducted after 3 times of passaging.

Drug treatment

The concentration of kaempferol (50.0 μg/ml) and H_2O_2 (100μmol/l) was applied to induce stress signals. The experiment was divided into seven groups: blank control cell, DMSO solvent control cells, oxidated cells (with 100μmol/lH_2O_2) drug-treated cells (with 100μmol/lH_2O_2+50.0 μg/ml, vitamin C, 100μmol/lH_2O_2+25.0 μg/ml kaempferol, 100μmol/lH_2O_2+50.0 μg/ml kaempferol, 100μmol/lH_2O_{2+}100.0 μg/ml kaempferol.

MTT assay to detect proliferation inhibition rate of HUVECs

A total of 0.25% trypsin (containing 0.02% EDTA) was used to digest the logarithmic HUVECs (Nijnik *et al*., 2007). After the digestion of the cells, cell density was adjusted to 2 × 10^5/ml in 100 μl/well and inoculated in 96 well plate. The cell culture plate was placed in a constant temperature incubator for 24 h. Afterwards, the cell culture supernatant was discarded and threated with drugs. These cells were cultured at 37 °C, and 5% CO_2 constant temperature incubator for 8, 15, 30 min. The supernatant was abandoned, treated with 20 μl MTT (5 μg/L) and incubated at constant temperature for 4 h. Cells were collected and centrifugation for 15 min at 2500 r/min. The supernatant was discarded and treated with DMSO (150 μl, 100 r/min oscillatory 10 min) and absorbance (A_{490}) was measured by enzyme labeling instrument. The inhibitory rate of cell proliferation was measured by the formula: (experimental group A_{490} / control A_{490}) × 100%.

Cell cycle detection

The logarithmic HUVECs were diluted into a single cell suspension with a concentration of 5× 10^5/ml. The cells were inoculated in 6-well cell culture plate with 2ml per well and cultured in 5% CO_2 and 37 °C incubator for 24 h. The culture medium in the original pore was discarded and different concentrations of 1.0ml solution were added to each hole. At the same time, the control group (supplemented with medium) was cultured for 24 hours. Cultured cells were maintained at 37 °C and 5%CO_2. The cells were digested with trypsin without EDTA and centrifuged with 1500 r·min^{-1}/min for 5 min. The centrifugation temperature was controlled at 2~8°C. The cells were washed with cold PBS twice (2~8°C,1500 r·min^{-1}/min centrifuged 5min) and the cells were collected. After adding 10μL PI staining solution, cells were incubated at 2~8°C for 5min, and were then gated using flow cytometry. The percentage of cells in G1 phase, S phase and G2 phase were calculated in different mock or treated cells.

Flow cytometry for detection of apoptosis in HUVECs

HUVECs were cultured in 6-well cell culture plate until logarithmic growth. The cells were divided into 7 groups. After treatment with HUVECs for 15min, the supernatant was discarded and the cells were collected. Using PBS to wash the cells twice (1000rpm centrifuged 10min), these were resuspended in 400 μL binding buffer. A volume of 5 μL Annexin V-FITC was added to each cell suspension and the suspension was incubated at 2-8°C for 15 min in the dark. Thereafter, a total of 10 μL propidium iodide was added and the suspension was incubated at 2-8°C for 5 min in the dark. Flow cytometry was performed within 1 h of the final step.

Flow cytometry to detect the number of HUVECs

The logarithmic HUVECs were adjusted to a single cell suspension of 5× 10^5/ml and were seeded into the 6-well cell culture plate (2.0ml per well). The cell were maintained at 5% CO_2 and 37 °C for 24 h. Drugs of different drug groups were measured and added to the cell culture plate (1.0ml per well) before maintaining them at 5% CO_2, 37 °C for 15min. The cells were digested with trypsin and centrifuged at 1500 rpm/min for 5 min. The centrifugation temperature was controlled at 2-8°C. The cells were washed twice with cold PBS. According to the reference manual, FITC-CD105/PE-CD62E labeled antibodies (Yao *et al*., 2009; Charpin *et al*., 2004) were added to the cell culture plate, cultured at room temperature for 20 min, the supernatant was discarded. The cells were washed twice with cold 250 μl of PBS and spun at 1000r/min for 5min before flow cytometry analysis.

Western blotting

The expression levels of p16 (Yan and Gong, 2011), p53, p21, Bax and Bcl-2 proteins were detected by Western blotting. Briefly, 100μmol/lH_2O_2+50.0 μg/ml kaempferol was used to treat cells in the 6-well cell culture plate. After the cells were cultured for 15min, the culture medium was discarded and the cells were washed with PBS twice. The RIPA lysis buffer (100 μl per well) was added, and cells were scraped into a single cell suspension. According to the requirements of BCA protein determination kit (whole protein extraction kit), the standard curve was drawn, and the protein concentration of the sample was determined. For each sample, 40 μg total protein was run on an SDS-PAGE gel (12%), followed by transfer to a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). Membranes were blocked for 1.5 h in 5% BSA (cat. no. A8806; Sigma-Aldrich; Merck KGaA) at 2-8˚C. The membranes were incubated with primary antibodies against p16, p53, p21, Bax or Bcl-2 for overnight at 4˚C. Subsequently, membranes were incubated with goat anti-rabbit immunoglobulin G-horseradish peroxidase conjugated antibodies (1:5,000; cat. no. A0208; Beyotime Institute of Biotechnology) at room temperature for 1 h, followed by chromogenic detection using an enhanced chemiluminescence reagent (cat. no. WP20005; Thermo Fisher Scientific, Inc.). The blots were scanned and analyzed using Gel-Pro-Analyzer 4.0 software (Media Cybernetics, Inc., Rockville, MD, USA), and the optical densities of the target bands were quantified relative to β-actin.

Statistical analysis

The data was analyzed using Graphpad Prism 5.0 software. The inhibitory rate of cell proliferation was as follows: $\bar{x}\pm s$ means that the average number of samples in multiple groups is compared by single factor variance analysis. The $p<0.05$ indicate a statistically significant difference.

RESULTS AND DISCUSSION

Proliferation inhibition in HUVECs

The cell death rate in oxidation-induced cell was significantly higher than that of the control cells ($p<0.001$), and the cell death rate in drug group was significantly lower than that of the oxidation group ($P<0.01$) (Table I). The MTT method was used to detect the inhibition rate in cells. According to the color of solution in each well in the cell culture plate, the antioxidant protection of cells with different concentrations of kaempferol was determined.

Cell cycle changes

Compared to oxidation-induced group, the percentage of G1 phase (Blomen and Boonstra, 2007), and S phase cells in vitamin C and drug-treated groups were decreased, and the percentage of G2 phase cells was significantly increased (Fig. 1, $p < 0.001$). Cell cycle (Witkiewicz *et al*., 2011) refers to the whole process that a cell goes through the beginning of one division to the end of the next. It is a complex and delicate regulatory process which is continuous and irreversible. Disorders of the cell cycle are associated with the development of many diseases (Guo *et al*., 2017). In this experiment, the cell cycle in the oxidation group changed to a large extent, and the use of kaempferol reversed the trend of cell cycle changes to a certain extent. The results indicated that kaempferol had a considerable antioxidant protection effect on HUVECs.

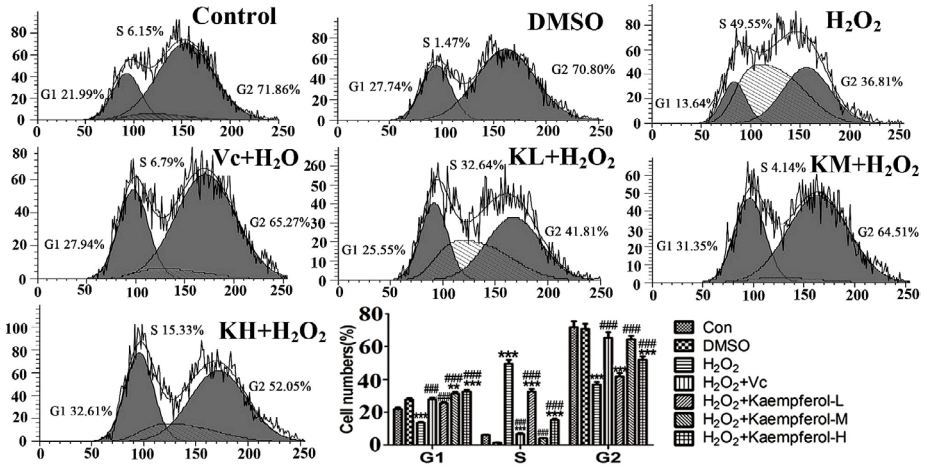

Fig. 1. Induction of glioma cell apoptosis by Kaempferol. Flow cytometry analysis of cell-cycle.
Note: Data are presented as the mean ± standard deviation, n = 6. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the control group; $^{\#\#\#}P < 0.001$ compared with the H_2O_2 group.

Table I. Kaempferol-induced protection of oxidized HUVECs cell proliferation ($n = 4$, $\bar{x}\pm s$).

Time	Apoptosis rate (%)						
	Control	DMSO	H_2O_2	H_2O_2+Vc	H_2O_2+K-L	H_2O_2+K-M	H_2O_2+K-H
8min	2.15±0.23	3.56±0.46	10.25±1.02	5.34±0.73	8.85±0.52	6.53±0.69	9.26±0.75
15min	3.26±0.42	5.27±0.59	21.84±3.52	9.26±0.61	15.26±0.95	12.57±2.19	17.21±1.09
30min	5.69±0.53	7.19±0.86	35.46±5.32	15.21±0.72	21.32±0.98	19.26±3.14	22.31±1.02

Note: $^{*}P < 0.05$; $^{**}P < 0.01$ and $^{***}P < 0.001$.

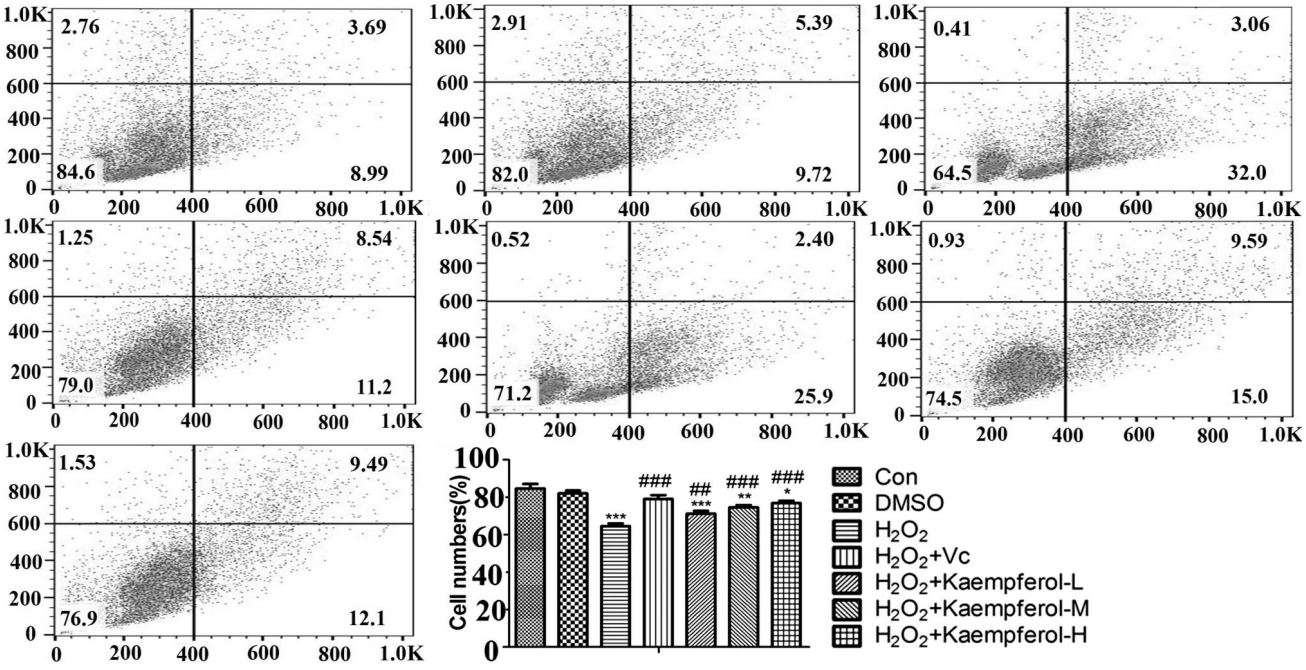

Fig. 2. Induction of glioma cell apoptosis by Kaempferol. Flow cytometry analysis of cell apoptosis.
Note: Data are presented as the mean ± standard deviation, n = 6. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the Control group; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the H_2O_2 group.

Apoptosis rate in HUVECs

The apoptosis rate of the oxidation-induced group was significantly higher than that of the control group, and the apoptosis rate in the drug-treated group was significantly lower than that of the oxidation group (Fig. 2, $P < 0.001$). We noticed that the apoptosis rate of cells in the oxidation group was significantly increased, which was reversed after the use of kaempferol. These results revealed that kaempferol can protect HUVECs from oxidative damage.

Apoptosis rate determined by labeling HUVECs

Compared with the control group, the number of HUVECs labeled with CD105/CD62E significantly lower in the oxidation group and CD105/CD62E cells in the drug-treated group was higher than in the oxidation group (Fig. 3, $P < 0.001$). Cell receptors are specific structures on the cell surface that can bind to certain biological substances (Dirsch *et al*., 2001) such as antigen receptor on the surface of the T cells (Rouard *et al*., 2000) and red blood cell receptor, and Fc receptor and C3b receptor on the surface of B cells. The special receptors on the surface of HUVECs are CD105 (Wang *et al*., 2020) and CD62E. In this experiment, these two receptors were selected for fluorescence labeling to detect the number of HUVECs. The results showed that the number of cells labeled with CD105/CD62E decreased in the oxidative group, while the number of HUVECs labeled with CD105/CD62E increased in the kaempferol-treated group. The results highlight the roles of kaempferol in protecting HUVECs from oxidative damage.

Expression of p16, p53 and p21 using western blotting

After oxidization for 15min, cells were treated with kaempferol (50.0μg/ml) and the protein expressions of p16,

p53, p21, Bcl-2 and Bax were detected. The expression of Bcl-2 in the oxidation group was significantly lower than that in the control group, while the expressions level of Bcl-2 in the drug group were significantly higher than those in the oxidation group. The expression of p16, p53, p21 and Bax in the oxidation group was significantly higher than that in the control group, while the expressions level of p16, p53, p21 and Bax in the drug group were significantly lower than those in the oxidation group (Fig. 4, $p < 0.05$).

Apoptosis is an active process, which involves the activation, expression and regulation of a series of cellular proteins. In this experiment, the levels of p16 (Cui *et al*., 2015; Stankovic *et al*., 2014), p53 (Taghavi *et al*., 2010), p21 and Bax proteins in the oxidative group were significantly increased, and the expression level of Bcl-2 was significantly decreased. Intriguingly, the kaempferol group reversed this trend. The results showed that kaempferol can protect HUVECs from the oxidative damage.

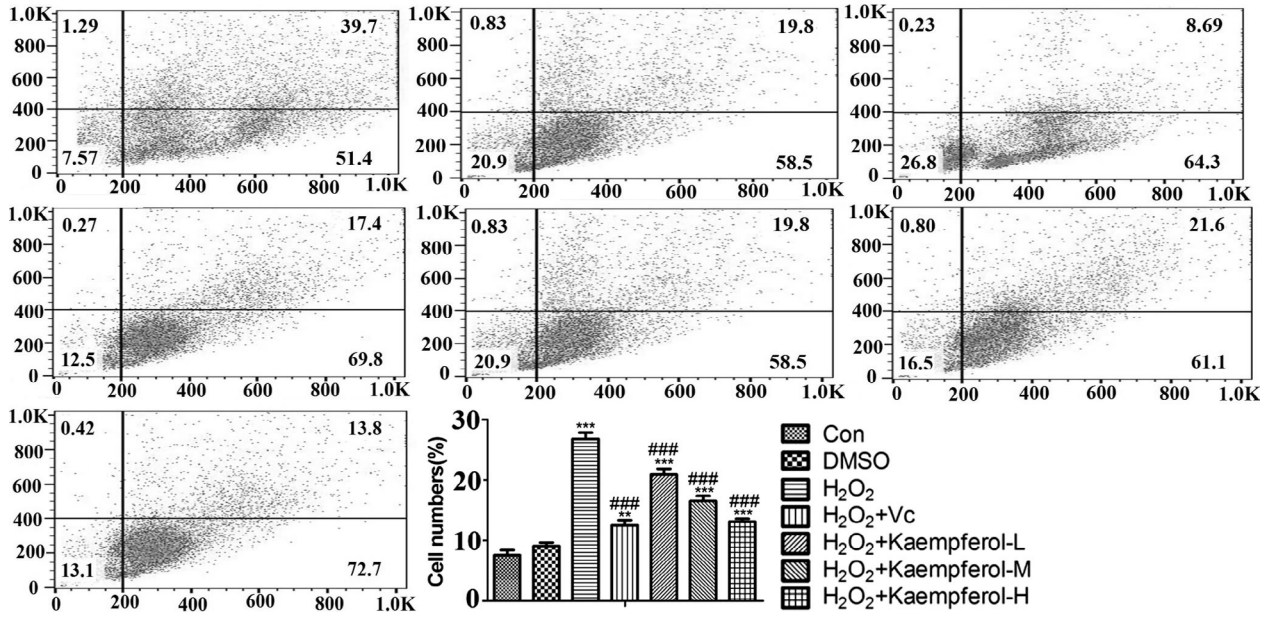

Fig. 3. Induction of glioma cell apoptosis by Kaempferol. Flow cytometry analysis of cell apoptosis.
Note: Data are presented as the mean ± standard deviation, n = 6. ***$P < 0.001$ compared with the Control group. $^{###}P < 0.001$ compared with the H_2O_2 group.

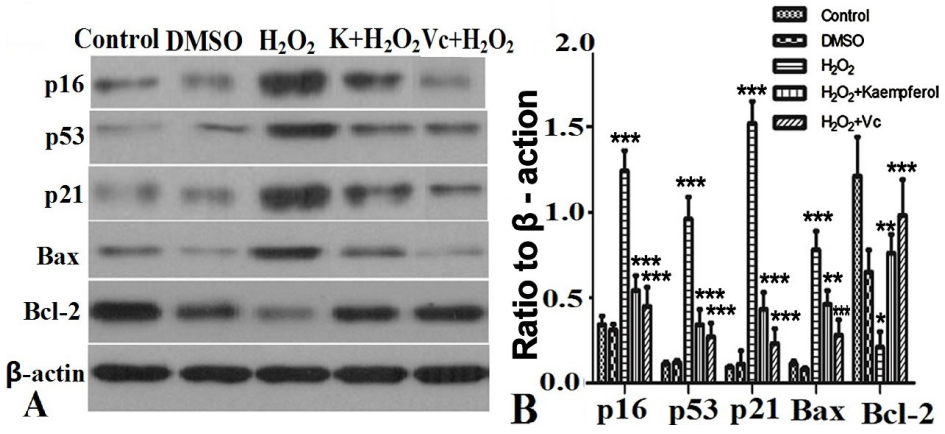

Fig. 4. Induction of glioma cell apoptosis by Kaempferol. (A.B) Western blot analysis of the expression of Bcl-2, Bax, p16, p53 and p21; β-actin was used as an internal.
Note: Data are presented as the mean ± standard deviation, n = 6. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

CONCLUSION

Our findings reveal that kaempferol inhibited oxidized HUVECs apoptosis, and protected cell cycle possibly by decreasing the expression level of p16, p53, p21 and Bax or by increasing the expression level of Bcl-2 proteins. These results demonstrate a potential therapeutic use of kaempferol as antioxidant agents in medicine. These finding underlay some of the key markers associated with the hydrogen peroxide induced oxidative damage and may guide future pharmaceutical interventions.

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Statement of conflicts of interest

The authors have declared no conflict of interest.

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